Characterization of Imp-L2 - a novel extracellular inhibitor of *Drosophila* insulin

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von

Basil Honegger

von

Wald (ZH)

Promotionskomitee

Prof. Dr. Ernst Hafen*

Prof. Dr. Konrad Basler

Prof. Dr. Peter Gallant

*Leitung der Dissertation

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Summary

Differences in size are often the most distinctive features between individuals and species. The size of an animal depends mainly on the size and number of the cells it contains. Therefore, the control of cell, organ, and body growth is of fundamental importance to the development of any organism. How cells know when they have to grow or divide, and when they have to stop growing, remains obscure. The evolutionarily conserved insulin receptor (InR) and Target of Rapamycin (TOR) signaling cascades have been found to integrate various signals like nutrients, growth factors, and oxygen levels to regulate protein synthesis, metabolism, reproduction, and lifespan. Deregulated activity of both pathways results in severe metabolic- and growth anomalies like diabetes, inflammation, and cancer. Since mutations in the two only known negative regulators, PTEN or TSC, of the InR/TOR cascades are implicated in the development of a wide variety of human tumors, all inhibitors of these pathways can be considered as putative tumor suppressors. Therefore, to better understand the development of cancer and to identify potential therapeutic targets, it is essential to identify all the gene products involved in the regulation of the two cascades.
This PhD thesis describes the functional characterization of a novel negative regulator of the insulin signaling pathway, the Imaginal morphogenesis protein-Late2 (Imp-L2). *Imp-L2* has been identified in an enhancer/promotor (EP) based overexpression screen searching for suppressors of the hyperplasic eye phenotype induced by ectopic expression of the InR in the developing eye. Imp-L2 is a secreted member of the immunoglobulin (Ig) superfamily, which inhibits growth non-autonomously by suppressing insulin signaling in *Drosophila*. Like its ortholog the putative vertebrate tumor suppressor insulin-like growth factor-binding protein-related Protein-1 (IGFBP-rP1), Imp-L2 binds at least one of the seven *Drosophila* insulin-like peptides (Dilps) and counteracts its activity, thereby providing a way of compensating hyperinsulinemia. Under adverse nutritional conditions, *Imp-L2* is upregulated and necessary for larval survival by attenuating insulin signaling activity. Additionally, *Imp-L2* is also induced under lowered oxygen levels (hypoxia). This indicates that Imp-L2 functions as a fast inhibitor of insulin activity under unfavorable environmental conditions.

Zusammenfassung

Unterschiedliche Grösse ist oft das auffälligste Merkmal zwischen einzelnen Individuen und Spezies, weshalb die Kontrolle von Zell-, Organ- und Körperwachstum von fundamentaler Bedeutung für die Entwicklung eines Organismus ist. Es ist jedoch unklar, woher die Zellen wissen, wann sie wachsen oder sich teilen sollten, und wann das Wachstum wieder gestoppt werden muss. Die beiden evolutionär konservierten Signalkaskaden, der Insulin Rezeptor (InR) und der Target of Rapamycin (TOR) Signalweg, integrieren verschiedenste Signale wie Nahrungsangebot, Wachstumsfaktoren und Sauerstoffangebot, um Proteinsynthese, Metabolismus, Reproduktion und Lebensdauer zu steuern. Deregulierte Aktivität beider Signalwege führt zu schweren Metabolismus- und Wachstumsanomalien wie zu Beispiel Diabetes, chronischen Entzündungen und Krebs. Da Mutationen in den einzigen zwei bekannten negativen Regulatoren der InR/TOR-Kaskade, PTEN und TSC, an der Entwicklung einer grossen Anzahl menschlicher Tumore beteiligt sind, können alle Inhibitoren dieser Signalwege als potentielle Tumor-Suppressoren betrachtet werden. Um die Entstehung von Krebs besser verstehen zu können und mögliche Therapien dagegen zu entwickeln, ist es deshalb essentiell, alle Gene zu identifizieren die an der Regulierung dieser zwei Signalkaskaden beteiligt sind.

In der vorliegenden Doktorarbeit wird die funktionelle Charakterisierung eines neuen negativen Regulators des Insulin-Signalweges, des Imaginal morphogenesis protein-Late2 (Imp-L2), beschrieben. *Imp-L2* wurde in einem enhancer/promotor (EP) Überexpressions-Screen gefunden, in welchem Suppressoren, des durch ektopische InR-Expression verursachten „Grosse-Augen“ Phänotyps gesucht wurden. Imp-L2 ist ein sekretiertes Mitglied der Immunoglobulin (Ig) Familie, welches das Wachstum in *Drosophila* nicht-autonom, durch Runterregulierung des Insulin Signalweges inhibiert. Wie sein Ortholog, Insulin-like growth factor-binding protein-related Protein-1 (IGFBP-rP1), ein potentielle Tumor-Supressor in Vertebraten, bindet Imp-L2 wenigstens eines der sieben *Drosophila* insulin-like peptides (Dilps) und hemmt dessen Funktion. Dadurch kann Imp-L2 stark erhöhte Insulin Pegel kompensieren. Unter ungünstigen Nahrungsbedingungen wird die Expression von *Imp-L2* induziert, was fürs Überleben der Larven und die nötige Abschwächung des Insulin Signals essentiell ist. Zusätzlich wird Imp-L2 auch unter tiefen Sauerstoff-Bedingungen (Hypoxische Bedingungen) raufreguliert. Dies alles deutet darauf hin, dass Imp-L2 als schneller Inhibitor der Insulin Aktivität unter ungünstigen Umweltbedingungen verwendet wird.

Introduction

Growth is determined by a wide variety of cues

Cell growth, defined as an increase in mass, is a highly regulated process, being subject to both temporal and spatial controls. It is usually coupled with cell division - an increase in cell number - to give rise to an organ or organism of characteristic size. Differences in size are often the most distinctive features between, but also within different species, yet we still know little about the genetic basis of size regulation (Conlon and Raff 1999). How is it possible that animals of the same class, like the two fishes whale shark (12.65m) and dwarf goby (8.6mm) vary 1500 fold in size? The size of an animal, organ, or appendage depends on the number and size of the cells it contains as well as on the amount of extracellular matrix and fluid. Although cell proliferation (increase in cell number controlled by cell division and programmed cell death) and cell growth (increase in cellular size) are often coupled processes, they must be considered separately, because cells can either grow to different sizes or divide without growth to produce larger numbers of smaller cells. The observation that individuals of the same species and genotype tend to grow to a highly predictable size suggests that growth is under genetic control. The size of a cell is largely proportional to the amount of DNA it contains (Stocker and Hafen 2000). In insects like *Drosophila*, cells of the so-called endoreplicative tissues grow by replicating their DNA without intervening mitosis thereby reaching huge sizes in comparison with epithelial cells, and ploidies from 16C to 2048C (Edgar and Orr-Weaver 2001).

However, growth and final body size are also influenced by external factors like temperature, nutrition or oxygen levels of the environment. For example, rearing flies under crowding, low oxygen (hypoxic), or starvation conditions results in a developmental delay and in the generation of tiny animals. Moreover, flies reared at lower temperature display enlarged body structures compared to control animals reared at higher temperature due to cell size increases (French et al. 1998; Azevedo et al. 2002).

Within the last thirty years a considerable amount of progress about the control mechanisms of the cell cycle has been made. However, the understanding of cell growth, especially how cells integrate extrinsic and intrinsic stimuli to adapt metabolism and growth, lagged behind.

Cell growth does not depend on progression through the cell division cycle, as mutations that block cell cycle progression tend not to arrest cell growth (Johnston et al. 1977; Weigmann et al. 1997; Neufeld et al. 1998). Moreover, some cells as nerve and muscle cells, grow mainly after they have permanently withdrawn from the cell cycle, others, such as *Drosophila* imaginal disc cells, grow before they enter the cell cycle (Madhavan and Schneiderman 1977). However, how cell growth is coupled with the cell cycle has remained elusive. A solution to couple these two processes directly might be to connect the synthesis of factors rate-limiting for cell cycle progression and the translational capacity. In yeast, the G1 cyclin Cln3p determines the critical growth-rate threshold for division. Due to the presence of an upstream open reading frame (uORF) in the mRNA of *Cln3p*, under adverse nutritional conditions, *Cln3p* is only poorly translated. Conversely, when nutrients are sufficiently abundant, the inhibiting effect of the uORF is overcome, and the cell enters S-phase (Polymenis and Schmidt 1997).

Two major regulators of the complex growth process, controlling both cell number and cell size, are the evolutionarily conserved insulin receptor (InR) and Target of Rapamycin (TOR) pathways. Both pathways are necessary to couple environmental factors such as nutrients or oxygen levels directly to the growth rate of a cell. The high evolutionary conservation of InR and TOR signaling in higher eukaryotes can also be used for medical research. Deregulation of insulin signaling causes severe diseases such as diabetes, cancer, and inflammation (Hill and Hemmings 2002; Bjornsti and Houghton 2004; Sansal and Sellers 2004). In fact, the tumor suppressor PTEN is frequently lost in a variety of cancer types (Cantley and Neel 1999; Simpson and Parsons 2001). Therefore, the identification of novel signaling components in model organisms, such as *C. elegans* and *Drosophila melanogaster*, might enhance the understanding of human diseases and eventually lead to novel therapeutic interventions.

The Insulin/IGF signaling pathway

Vertebrate insulin and insulin-like peptides

The insulin/insulin-like growth factor (IGF) signaling (IIS) cascade is an ancient pathway that is well conserved between vertebrates and invertebrates. Extensive studies of this pathway

have shown that IIS is split into complementary and interacting subsystems that govern growth, metabolism, reproduction, and longevity (Nakae et al. 2001; Saltiel and Kahn 2001). Insulin, which is produced by β-cells of the endocrine pancreas, on one hand increases the storage of glucose, fatty acids, and amino acids in adipose tissue and muscle, and on the other hand inhibits hepatic glucose production. Thus, insulin serves as the primary regulator of blood glucose concentration, which remains always in a narrow range between 4 and 7mM in normal human individuals (Saltiel and Kahn 2001). Glucose is metabolized by all tissues, but is a critical metabolic fuel for the nervous system including the brain (McCall 1993). Since the brain is unable to metabolize fatty acids, under physiological conditions, it depends almost exclusively on the metabolism of glucose (~85% of which is terminally oxidized) for its energy production. Glucose oxidation normally accounts for virtually all of the oxygen consumed by the brain; the brain respiratory quotient is nearly 1.0 (Sokoloff 1989). Thus, even though other fuels, such as ketone bodies (water-soluble fatty acid equivalents that are produced by the liver, namely acetone, acetoacetate, and hydroxybutyrate), can partially substitute as a brain energy source when their circulating levels rise high enough for them to enter the brain in quantity, as ketone bodies do during fasting (Owen et al. 1967; Hasselbalch et al. 1995), availability of glucose is critical for the survival of the brain, and therefore for the whole individual. Impaired insulin signaling leads to a massive increase of blood glucose levels resulting in a pathological condition called Diabetes mellitus. The chronic hyperglycemia resulting from diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels. Diabetes mellitus is a fast spreading disease that currently afflicts around 150 million people world wide and is predicted to increase to 300 million people by the year 2025 (Green et al. 2003). According to the Report of the Expert Committee on the Diagnosis and Classification of Diabetes mellitus (Gavin et al. 2000), diabetes can be classified etiologically into four major types: type I diabetes, type II diabetes, other types, and gestational diabetes. However, type I and type II constitute the two major forms and afflict almost 90% of the total diabetic population. Type I Diabetes mellitus, previously termed Insulin Dependent Diabetes Mellitus (IDDM), accounts for less than 10% of all cases. It results from a complete autoimmune destruction of the β-cells in the pancreas, caused by either environmental or genetic factors. Type II diabetes, formerly termed Non-Insulin Dependent Diabetes Mellitus (NIDDM), results from a combination of resistance to insulin action and an inadequate compensatory insulin secretory response by the pancreatic β-cells. An estimated 100 million people world-wide of a wide range of ethnic groups and all social and economic levels suffer from this type

of diabetes (Green et al. 2003). Epidemological and twin studies indicate a polygenic predisposition for type II diabetes (Froguel et al. 1993). However, environmental factors such as obesity and sedentary life style can aggravate genetically determined insulin resistance. Besides its role as main regulator of blood glucose, insulin also stimulates cell growth and differentiation by promoting the storage of substrates in fat, liver and muscle by stimulating lipogenesis, glycogen-, and protein synthesis, and inhibiting lipolysis, glycogenolysis and protein breakdown (Saltiel and Kahn 2001). However, in vertebrates, circulating IGFs, which are phylogenetically closely related to insulin (Plisetskaya 1989), are the key regulators of IIS-dependent growth effects. Circulating IGFs are mainly produced by the liver upon induction of growth hormone (GH) (Lund 1999) and are essential for regulating growth and body size both pre-natally (IGF-1 and IGF-2) and post-natally (IGF-1)(Efstratiadis 1998; Nakae et al. 2001). Because many components from the IIS pathway have been isolated as retroviral oncogenes and as tumor-suppressor genes from mammalian systems, it has been suggested that deregulation of the IIS system can lead to cancer (Valentinis and Baserga 2001; Vogt 2001). Proto-oncogene activation and loss-of-tumor-suppressor gene function lead to the initiation and progression of a variety of cancers, yet the basis for their tumorigenicity is often unknown because multiple genetic lesions are required to undermine the anti-proliferative checkpoints of a cell (Vogelstein and Kinzler 1993; Hanahan and Weinberg 2000).

Apart from insulin and the IGFs, the insulin superfamily consists of at least five more members, namely relaxin, insulin-3, -4, -5, and -6 (Nakae et al. 2001). Relaxin, a hormone important for the growth and remodeling of reproductive and other tissues, does probably not signal through the canonical tyrosine kinase receptors (see below), but by the two G-protein coupled receptors LGR7 and LGR8 (Hsu et al. 2002). Insulin-3 is responsible for testicular descent through virilization, outgrowth of the embryonic gubernaculum, and gets specifically downregulated by estrogens (Nef et al. 2000). Insulin-4 is believed to play an important role in trophoblast development and regulation of bone formation (Chassin et al. 1995; Laurent et al. 1998). Insulin-5 is expressed in the hypothalamus and the pituitary gland, and is probably involved in thymic development and regulation (Conklin et al. 1999; Dun et al. 2006). Insulin-6 is known to be expressed at high levels in testes, but its function is so far unknown (Lok et al. 2000).

All in all, there exist at least eight different insulin-like proteins in vertebrates, which carry out a wide variety of functions. How exactly the IIS system regulates, coordinates, and integrates all these processes remains at a rudimentary stage of understanding. One approach

to understand this orchestration is to utilize the fruit fly *Drosophila melanogaster*, in which IIS is, despite the ~700 million year evolutionary divergence, astonishingly well conserved (Oldham and Hafen 2003).

The IGF-Binding Protein (IGFBP) Superfamily

In biological fluids of vertebrates, IGFs are normally bound to IGFBPs. There are, at present, six well-characterized mammalian IGFBPs, designated IGFBP-1 through -6, which have higher affinities for IGFs than for the IGF-1 receptor (IGF-1R) and are present in molar excess relative to the sum of the concentrations of IGF-1 and -2 (Hwa et al. 1999). Therefore, the variables that control the molar concentration of each binding protein in extracellular fluids are important determinants of IGF action. IGFBP-3, which the most abundant of the IGFBPs, transports >75% of serum IGF-1 and -2 (Burger et al. 2005). The functions of the IGFBPs can be divided into four major areas. The first is their role as transport proteins for the IGFs in plasma, which also serve to control the efflux of IGFs from the vascular space. A second function of the IGFBPs is to regulate the half-lives and metabolic clearance rates of IGF-1 and -2. The third function is to provide a means of tissue- and cell-type-specific localization of the IGFs, and the fourth is to directly modulate the interaction of IGFs with their receptors (Clemmons 1999). Although a huge amount of both *in vitro* and *in vivo* studies have been conducted, it still remains elusive whether IGFBPs inhibit or augment IGF actions. A possible explanation is that the different cell lines used react in distinct ways to the presence of IGF/IGFBP complexes, because some secrete for instance certain proteases known to cleave IGFBPs, thereby decreasing their affinity for IGFs. Another explanation lies within the dual nature of the IGFBPs, which on one hand binds IGFs better than the receptors do, and on the other hand increases the half-life of the IGFs, thereby prolonging their presence in the system. Therefore, IGFBPs have the potential to either restrict or increase IGF activity. The primary structures of mammalian IGFBPs contains three distinct domains of roughly equivalent sizes: the conserved N-terminal domain, the highly variable midregion, and the conserved C-terminal domain. Overall, the human IGFBPs share approximately 36% similarity, although alignment of e.g. the conserved N-terminal domain shows significantly higher similarities, namely 56% (Hwa et al. 1999). In the 80-93 amino acid containing N-terminal region of IGFBP1-5 all the 12 cysteines are fully conserved, whereas in IGFBP-6, 10

of the 12 cysteines are invariant. The high number of cysteines within such a small domain suggests that this domain is highly structured, with a maximum of 6 disulfide bonds formed (5 in the case of IGFBP-6)(Hwa et al. 1999). Additionally to the conserved cysteines, the N-terminal domain contains a local motif (GCGCCxxC) that is highly conserved among all IGFBPs and only present in vertebrates. However, the function of this motif is unknown. From its high degree of conservation, it is assumed to be important for the interactions with IGFs, though (Hwa et al. 1999). The midregion of human IGFBPs shares less than 15% similarity, is believed to act structurally as a hinge between the N- an C-terminal domains, and contrary to those domains contains posttranslational modification (phosphorylation, glycosylation) sites. The C-terminal domain of the IGFBPs is again highly conserved and shares approximately 34% similarity. It contains six cysteines, which are all strictly conserved. To efficiently bind IGF with high affinity, the presence of both the N- and the C-terminal domains are essential (Hwa et al. 1999).

Recently, an additional ten potential members of the IGFBP superfamily have been identified through computer analysis of GenBank sequence data, on the basis of the presence of the IGFBP motif, GCGCCxxC (Rosenfeld and Oh 1998; Burger et al. 2005). These proteins have considerably reduced binding affinities for the IGFs and are named as IGFBP-related proteins (IGFBP-rPs-1 to -10). IGFBP-rP-1, the first of these proteins to be found, was originally identified as a cDNA, termed mac25 (later also IGFBP-7/T1A12/TAF/AGM/PSF/PGI2), that was overexpressed in normal leptomeningeal cells compared to meningiomas (Murphy et al. 1993). In IGFBP-rP-1 the first 112 amino acids show approximately 50% identity to IGFBP-1, but distally, the sequence diverges completely. Interestingly, the conserved region spans exactly the first two exons. IGFBP-rP-1 is distinct from other IGFBP-rPs in that it can bind, additionally to binding IGF, strongly to insulin (Yamanaka et al. 1997). Although the affinity of IGFBP-rP-1 to IGFs and insulin is similar, its insulin:IGF binding ratio is 500-fold higher than that of IGFBP-1 to 6 (Yamanaka et al. 1997). Thus, IGFBP-rP-1 can compete with the InRs for binding of insulin, whereas for example IGFBP-3 cannot. In addition to binding insulin, IGFBP-rP-1 has also been shown to bind to activin, a member of the TGF-β superfamily of growth factors (Kato 2000). A study examining IGFBP-rP-1 protein expression in 60 primary breast cancers using immunohistochemistry revealed that 12 normal and benign breast tissues had strong IGFBP-rP-1 expression, 16 ductal carcinomas *in situ* showed weak IGFBP-rP-1 levels, and the invasive carcinomas were all negative for IGFBP-rP-1 (Burger et al. 1998). This is indicating that IGFBP-rP-1 acts as a tumor suppressor.

Apart from its function in mediating cellular proliferation, IGFBP-rP-1 also regulates adhesion, angiogenesis, and stimulates prostacyclin synthesis (Burger et al. 2005). Additionally, IGFBP-rP-1has been found to be one of the major genes implicated in human endometrial (the endometrium is the inner uterine membrane, which is developed in preparation for the implantation of a fertilized egg, upon its arrival in the uterus) receptivity, folliculogenesis, as well as growth development and regression of the *corpus luteum* in higher mammals (Wandji et al. 2000; Dominguez et al. 2003; Casey et al. 2004). The role of IGFBP-rP-1 in le reproductive system is further supported by the knock-out mouse of IGFBP-rP-1 (Burger et al. 2005). Mice lacking IGFBP-rP-1 are viable, but at the age of 5-8 months, significant changes in the histology of the ovaries, muscle tissue, and the liver have been observed (Burger et al. 2005). In particular, gross abnormalities in the *corpus luteum* have been described (Burger et al. 2005).

The acid-labile subunit (ALS)

As already mentioned above, in adult animals, plasma IGFs form higher molecular weight complexes with specific IGFBPs (IGFBP-1 to -6). These complexes are referred to as ~50kD binary complexes consisting of IGF-1 or IGF-2 and one IGFBP, or as 150kD ternary complexes consisting of one molecule each of IGF-1 or IGF-2, IGFBP-3 or IGFBP-5, and a 85kD glycoprotein, the acid-labile subunit (ALS) (Boisclair et al. 2001; Domene et al. 2005). ALS has no affinity for free IGF-1 or IGF-2, and very low affinity for uncomplexed IGFBP-3. However, it readily binds to binary complexes of IGF and IGFBP-3 (Baxter and Martin 1989; Twigg and Baxter 1998). Virtually no free (unbound) IGFs are present in the circulation of vertebrates (Jones and Clemmons 1995; Stewart and Rotwein 1996). Unlike free IGFs and IGFs bound to the ~50kD binary complexes, which can cross the vascular endothelium, formation of the ternary complexes restricts the IGFs to the circulation, prolongs their half-lives and allows them to be stored at high concentration in plasma to facilitate their endocrine actions and to minimize their local effects due to their intrinsic insulin-like activities such as hypoglycemia (Zapf 1995). In adult animals, serum IGFs reach concentrations that are 1000 fold that of insulin (Boisclair et al. 2001), which would be high enough for free IGFs to also stimulate the InR. Thus, ALS is a critical component that contributes to the development of this large IGF reservoir by extending its half-live from 10min when in free form, and 30-

90min when in binary complexes, to more than 12h when bound in ternary complexes (Guler et al. 1989; Zapf 1995). Under acidic conditions, the ternary complex is irreversibly dissociated and free IGF is liberated (Martin and Baxter 1986). Neutralization after acidification allows for the re-association of IGF and IGFBP, but does not reconstitute the ternary complex (Murphy 1998).

ALS, synthesized exclusively by the liver, is predominantly stimulated by growth hormone (GH), as are both IGF-1 and IGFBP-3 (Boisclair et al. 2001). The ALS protein contains 578 amino acids with seven potential N-glycosylation sites. Further it has an unique structure with 20 leucine-rich repeats of 24 amino acids, each with a similar consensus sequence. This structure is a general mediator of protein-protein interactions, and probably accounts for the high affinity of ALS for the binary IGFBP-IGF complexes (Clemmons 1999). A variety of conditions have been shown to reduce serum ALS in rats and humans. They include fasting, undernutrition, and catabolic diseases such as diabetes, burn injury and cirrhosis (Boisclair et al. 2001). Negative regulation of ALS synthesis occurs at both transcriptional and post-transcriptional levels. Ablation of ALS has no effect on fetal growth in both the ALS-KO mice and the ALS-deficient patients. However, a modest reduction in post-natal growth in the null ALS mice (13%) and in the ALS-deficient patients was observed (Domene et al. 2005). Interestingly, ALS deficiency results in a dramatic reduction in circulating IGF-1 and IGFBP-3 concentrations (without affecting their synthesis) compared to the wild-type situation (in mice the reductions are 62% and 88% respectively)(Boisclair et al. 2001). In contrast to the ALS-KO mice, human patients lacking ALS develop insulin resistance (Domene et al. 2005). All in all, ALS plays a major role in generating the large IGF pool in the circulation, which seems to be partly necessary for post-natal growth.

Recently, an ALS ortholog has also been identified in *Drosophila* (dALS)(Colombani et al. 2003). dALS is expressed in the Drosophila liver analog, fat body, and in seven distinct median neurosecretory cells (m-NSCs) of both larval brain hemispheres, that also express four of the *Drosophila* insulins (see below). Like in vertebrates, the expression of dALS is highly sensitive to starvation and amino acid restrictions (Colombani et al. 2003). Whether dALS functions in a similar way to vertebrate ALS, remains to be tested.

Insulin signaling in *Drosophila*

The presence of an insulin-like hormone in insects has been already proposed 30 years ago (Seecof and Dewhurst 1974; Normann 1975; Duve et al. 1979). Since then, molecular and genetic analysis in *Drosophila* has demonstrated that the IIS system is not only present in insects, but also extremely well conserved. IIS in *Drosophila* controls growth, metabolism, reproduction, and longevity (Garofalo 2002). While vertebrates contain four different receptor tyrosine kinases for the insulin family peptides, the insulin receptor (InR), the IGF-1 receptor (IGF-1R), the IGF-2 receptor (IGF-2R), and the insulin related receptor (IRR), in the *Drosophila* genome only a single insulin receptor is present (Fernandez et al. 1995; Chen et al. 1996). The *Drosophila* insulin receptor (dInR), like its mammalian counterparts, comprised two α and two β subunits, with a tyrosine kinase domain in its β subunit that is activated upon insulin binding (Fernandez-Almonacid and Rosen 1987). Albeit the evolutionary distance between flies and humans, the InRs are, especially around the kinase domain, highly conserved, and dInR also binds mammalian insulin with reasonably high affinity (Garofalo 2002). However, the signaling mechanism of dInR may differ slightly from that of its mammalian homologs, as it possesses a carboxy-terminal extension adjacent to its kinase domain that contains various tyrosine phosphorylation sites. Autophosphorylation of this carboxy-terminal extension is predicted to allow dInR to recruit downstream signaling molecules directly without the need for intermediate adaptor proteins. *dInR* has an essential function during normal insect development, because strong *dInR* mutations are recessive embryonic lethal (Fernandez et al. 1995; Chen et al. 1996). Hypomorphic *dInR* combinations produce viable adults that show a dramatic reduction in overall body size, and display increased longevity (Chen et al. 1996; Brogiolo et al. 2001; Tatar et al. 2001). Ablation of the *InR* in mice leads to only a slight growth retardation (10% weight decrease), but to the development of severe hyperglycemia accompanied by an extreme elevation of insulin levels (Accili et al. 2001). In addition to the control of size and metabolism in vertebrates, the insulin receptor family, comprising InR, IGF-1R, and IRR, is required for the appearance of the male gonads and thus for male sexual differentiation (Nef et al. 2003). XY mice that are mutant for all the three receptors develop ovaries and show a completely female phenotype (Nef et al. 2003). So far there are no hints about an implication of dInR in the development of the male sexual differentiation in *Drosophila*, but viable combinations of *dInR* alleles display female sterility (Chen et al. 1996).

Recently, seven putative ligands, the *Drosophila* insulin-like peptides (Dilps), of the dInR, with homology to human insulin have been identified in *Drosophila* (Brogiolo et al. 2001). When overexpressed ubiquitously, all *dilps* promote growth (Ikeya et al. 2002). However, direct binding of any of the Dilps to the dInR has not been demonstrated yet, although Dilp2, the closest ortholog of mammalian insulin and the most potent growth inducing insulin in *Drosophila*, has been shown to genetically interact with the *dInR* (Brogiolo et al. 2001). The different *dilp* genes have distinct expression patterns, suggesting distinct functions (Brogiolo et al. 2001). Four of the seven dilps are expressed in seven cells of each hemisphere of the larval brain that might correspond to neurosecretory cells (Brogiolo et al. 2001; Cao and Brown 2001; Rulifson et al. 2002). Targeted ablation of these median neurosecretory cells (m-NSC) causes growth retardation, developmental delay and elevated carbohydrate levels in the larval hemolymph (the insect equivalent of blood)(Rulifson et al. 2002). All these effect can be reversed by the ectopic expression of a *dilp2* transgene (Rulifson et al. 2002). Thus, like its mammalian counterpart, *dilp2* regulates growth and carbohydrate levels of the hemolymph. Since no mutants for any of the *dilps* exist, the m-NSC ablation phenotype provides the only hint at how a *dilp* loss-of-function phenotype could look like.

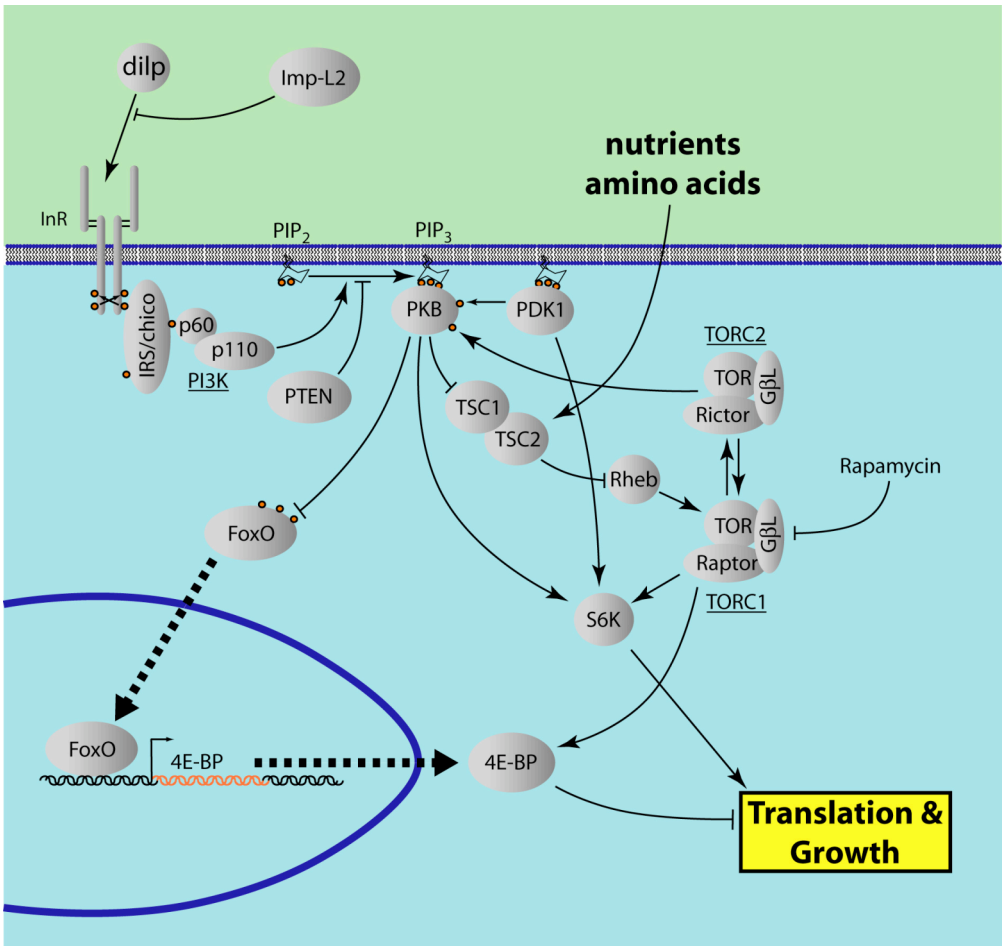


Figure 1: The insulin signaling pathway in Drosophila.

Intracellular insulin signaling

Upon binding of the ligand, the InR undergoes a conformational change, which results in the autophosphorylation on several of its cytosolic tyrosine residues in the β-subunit (Ullrich and Schlessinger 1990). Once activated, the receptors transduce signals downstream by phosphorylating various intracellular substrates, including insulin receptor substrate (IRS) proteins. IRS proteins bind via their phosphotyrosine-binding (PTB) domains to autophosphorylated tyrosine residues in the juxtamembrane regions of the activated receptors (Leevers and Hafen 2004). IRS proteins lack intrinsic catalytic activities but are composed of multiple interaction domains and phosphorylation motifs. At least three IRS proteins occur in humans and mice, including IRS-1 and IRS-2, which are widely expressed, and IRS-4, which is limited to the thymus, brain, and kidney and possibly β-cells (Uchida et al. 2000). Rodents also express IRS-3, which is largely restricted to adipose tissue and displays activity similar to IRS-1. However, this short ortholog might not occur in humans (White 2002). *Drosophila* contains just a single ortholog of the IRS proteins termed chico. Like its mammalian homologs chico contains, in addition to the PTB domain, an N-terminal pleckstrin homology (PH) domain and several phosphotyrosine motifs that serve as docking sites for Src-homology 2 (SH2) domain containing proteins. Two of these motifs (YxxM motifs) fit, after phosphorylation by the dInR, the SH2 binding site of the p60 (p85 in mammals) adaptor/regulatory subunit of the class I_A Phosphoinositide 3-kinase (PI3K). Through the binding of PI3K to chico, its catalytic subunit Dp110 gets activated (Weinkove et al. 1997; Weinkove et al. 1999). In Drosophila, it has also been shown that p60 is also able to directly bind to the InR, circumventing the chico adaptor protein (Oldham and Hafen 2003). Another phosphotyrosine motif of chico corresponds to the consensus binding site (YxN) of the GRB2/DRK protein, which activates the Ras-mitogen-activated protein kinase (MAPK) pathway in mammals (White 2002). In vertebrates, this binding of GRB2/DRK to chico represents a connection between the InR and the Ras-MAPK pathways. However, in *Drosophila* at the level of the IRS protein, no connection between insulin and the RAS/MAPK pathways could be established so far.

Conversely to mammals, which contain four class I_A PI3Ks, p110α, p110β, p110γ, and p110δ (Vanhaesebroeck et al. 2001), *Drosophila* possesses a single copy, dp110. Through the association with the phosphorylated chico, but also dInR, dp110/p60 heterodimers are

recruited to the plasma membrane, where dp110 gets access to its phosphoinositide substrate, phosphatidylinositol-4,5 bisphosphate (PIP_2). Via its lipid kinase domain, dp110 converts PIP_2, via phosphorylation of the 3' hydroxyl group of its inositol ring, to the critical second messenger phosphatidylinositol-3,4,5 trisphosphate (PIP_3) (Leevers and Hafen 2004). Elevated levels of PIP_3 transduce signals downstream by binding to molecules with PIP_3-binding pleckstrin-homology (PH) domains, thereby inducing their re-localization to the plasma membrane and/or conformational changes.

The activity of class I_A PI3Ks is counteracted by the phosphoinositide phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10). PTEN is a lipid phosphatase that specifically removes phosphates at the D3 position of the PIP_3 inositol ring (Maehama and Dixon 1998; Goberdhan et al. 1999; Huang et al. 1999; Gao et al. 2000). Through the dephosphorylation of PIP_3, PTEN has a very important role in limiting growth. Immortalized embryonic fibroblasts or embryonic stem cells lacking PTEN display a two to three-fold increase in PIP_3 levels (Hafen 2004). Loss of the tumor suppressor PTEN is a frequent event in the development of a wide variety of human cancers. PTEN germline mutations cause hereditary cancer predisposition syndromes like the Cowden-, Lhermitte-Duclos-, Bannayan-Zonana-, and the Proteus syndrome, which are all diseases associated with increased cancer incidence (Sulis and Parsons 2003). Thus, the levels of PIP_3 are under tight control, because any deregulation results in abnormal growth behavior.

In mammals, a number of signaling molecules possess PIP_3-binding PH domains, and hence have the potential to be regulated by PI3K. However, in *Drosophila*, two serine/threonine kinases have emerged as key downstream targets: the *Drosophila* homologs of phosphoinositide-dependent kinase 1 (dPDK1) and its substrate, the protein kinase B (PKB, also known as Akt)(Leevers and Hafen 2004). PKB is mainly cytosolic in unstimulated cells, but upon IIS activity is recruited to the plasma membrane by binding with its PH domain to PIP_3. Phosphorylation of two sites, Thr308 and Ser473, is required for rendering PKB fully active. Once recruited to the cell membrane, PKB co-localizes with PDK1, which has a significantly higher affinity for phosphoinositides (PIs). However, the PIs themselves have no effect on the catalytic activity of PDK1 (Currie et al. 1999). PDK1 is the kinase, which phosphorylates Thr308 in PKB after its translocation to the membrane. Binding of PKB to PIP_3 induces a conformational change in PKB, exposing its activation loop. This event makes Thr308 in PKB accessible for phosphorylation by PDK1. The responsible kinase phosphorylating Ser473 has remained elusive for a long time. However, it has been recently shown that in *Drosophila* and human cells, a complex consisting of the TOR kinase and its

associated proteins rictor and GβL (see also below) are not only directly phosphorylating PKB at Ser473, but also facilitate Thr308 phosphorylation by PDK1 (Sarbassov et al. 2005). Activated PKB then detaches from the membrane and translocates to the cytosol and nucleus, where it phosphorylates target proteins. In addition to phosphorylating PKB, PDK1 also directly activates the p70 S6 kinase (S6K, see also below)(Rintelen et al. 2001; Radimerski et al. 2002).

Downstream of PKB

PKB is one of the key effectors of the IIS signaling pathway, regulating growth, cell cycle, survival and nutrient metabolism. While vertebrates contain three copies of PKB proteins (Akt1-3), *Drosophila* contains a single one. The fact that reduced PKB activity is sufficient to rescue PTEN-induced lethality in *Drosophila* underscores its importance in transducing the insulin signal (Stocker et al. 2002). Therefore, most, if not all pathway activity is mediated through PKB in *Drosophila*. In vertebrates, over 50 proteins have been identified as putative PKB targets (Whiteman et al. 2002; Hanada et al. 2004). Amongst this growing list of substrates phosphorylated by PKB are the metabolic enzymes glycogen synthase kinase 3 (GSK-3) and 6-phospho-2-kinase, proteins involved in cell survival such as BAD, transcription factors of the Forkhead family, especially forkhead box O (FoxO), and the tumor suppressor protein Tuberous sclerosis 2 (TSC2)(Hafen 2004). In *Drosophila*, genetic and biochemical studies have identified two critical targets of PKB, namely the FoxO subfamily and TSC2. By phosphorylating TSC2, PKB stimulates the TOR pathway (see below). FoxO factors are insulin sensitive transcription factors regulating a variety of processes including differentiation, metabolism, stress resistance, proliferation, and survival. FoxO proteins contain an N-terminal DNA-binding domain and a C-terminal trans-activation domain. The FoxO DNA-binding domain is flanked by several conserved PKB phosphorylation sites, and its function is antagonized by PKB phosphorylation (Leevers and Hafen 2004). While Drosophila contains a single FoxO ortholog, the mammalian genome comprises four members of the FoxO subfamily, all of which contain several conserved PKB phosphorylation sites. PKB-mediated phosphorylation of FoxO inhibits their function by generating 14-3-3 protein-binding sites, thereby leading to the accumulation of 14-3-3-bound FoxO in the cytoplasm (Brunet et al. 1999). In the absence of PKB activity, FoxO is dephosphorylated, enters the

nucleus, and activates target genes (Hafen 2004). Although FoxO is the only known transcriptional read-out for insulin signaling so far, its function, unlike PTEN, is not essential for development and organismal growth control under normal culture conditions in *Drosophila* (Junger et al. 2003). The use of microarrays to identify putative FoxO targets revealed genes involved in the stress response, such as *cytochrome P450*, and the gene encoding the translational inhibitor, *Drosophila 4E-BP* (for *eukaryotic translation initiation factor 4E* (*eIF4E*) *binding protein*)(Junger et al. 2003). The *4E-BP* gene encodes a translational repressor and was initially identified in *Drosophila* as the immune-compromised Thor mutant in a genetic screen for genes involved in the innate immune response to bacterial infection (Rodriguez et al. 1996; Bernal and Kimbrell 2000). 4E-BP binds and inactivates the translation initiation factor eIF4E, which participates in the formation of a functional translation initiation complex. Positive transcriptional regulation of 4E-BP by FoxO, which corresponds to negative transcriptional regulation by insulin, would be a complementary mechanism of translational regulation.

Recently, also another member of the forkhead transcription factor family, Foxa2, has been found to be strongly regulated by insulin signaling in vertebrates (Wolfrum et al. 2004). Like FoxO, Foxa2 is inactivated by insulin and feeding, but surprisingly, Foxa2 is far more sensitive to insulin signals than FoxO, and consequently is turned off even in insulin resistant states, whereas FoxO is not (Wolfrum et al. 2004). Additionally, a mutant form of Foxa2, that is no longer regulated by IIS, expressed in the livers of diabetic mice reversed the hepatic steatosis (abnormal accumulation of certain fats in the liver) and improved insulin sensitivity, both phenotypes associated with insulin resistance and diabetes (Wolfrum et al. 2004). To date, the *Drosophila* ortholog of Foxa2 has not yet been identified. However, the only known forkhead family member containing, like Foxa2, a putative PKB phosphorylation site is the forkhead (fkh) protein. It remains to be tested, if fkh is a true target of PKB, and if it functions in a similar way as Foxa2.

TSC2 (tuberin, first named gigas in the fly), the second target of PKB, forms a complex with TSC1 (hamartin) and acts as a negative regulator of growth in *Drosophila*, and as a tumor suppressor in mammals. Mutations in human *TSC1* and *TSC2*, which affects 1 in 6000 individuals at birth (Cheadle et al. 2000; Li et al. 2004), are associated with heritable forms of tuberous sclerosis, a disease characterized by benign tumors (hamartomas) in the brain and other tissues (Montagne et al. 2001; Kandt 2002). In *Drosophila*, mutations in *dTSC1* and *dTSC2* have been identified in screens for genes that suppress growth. Loss of *dTSC1* or *dTSC2* accelerates growth and cell division, whereas co-expression of these two genes slows

growth and cell-cycle progression in a cell-autonomous fashion (Gao and Pan 2001; Potter et al. 2001; Tapon et al. 2001). Activated PKB phosphorylates TSC2 and thereby disrupts the TSC1/2 complex both in *Drosophila* and mammalian cells (Inoki et al. 2002; Potter et al. 2002). However, the significance of TSC2 phosphorylation by PKB may vary depending on the physiological context. In *Drosophila*, a TSC2 variant that cannot be phosphorylated rescues the lethality of a TSC2 mutant (Dong and Pan 2004), indicating that during fly development, PKB phosphorylation of TSC2 is not essential. Recent genetic and biological evidence suggests that TSC1/2 regulates growth by acting as a GTPase-activating protein (GAP) for the small GTPase Rheb (Ras homolog enriched in brain) (Garami et al. 2003; Saucedo et al. 2003; Stocker et al. 2003; Zhang et al. 2003). By acting as GAP toward Rheb, TSC2 decreases the GTP-bound, and thus active form of Rheb. Active Rheb stimulates growth and translation by binding directly to the kinase domain in TOR and activates TOR in a GTP-dependent manner (Long et al. 2005). However, it is still unclear whether GTP-loading of Rheb is required for TOR binding or for a subsequent TOR-activation step (Wullschleger et al. 2006).

The dual role of TOR

TOR was originally identified in yeast by mutations that are resistant to the growth inhibiting effects of the antifungal and immunosuppressant drug rapamycin (Heitman et al. 1991). This study also demonstrated that rapamycin inhibits the function of TOR by forming a complex with FK506-binding protein-12 (FKBP-12), which binds to a conserved region located immediately upstream of TOR's kinase domain. TOR is a member of the PI kinase-related kinase (PIKK) family of proteins that structurally resemble PIKs but possess Ser/Thr rather than lipid kinase activity (Leevers and Hafen 2004). To date, every eukaryote genome examined (including yeast, algae, slime mold, plants, worms, flies, and mammals) contains a *TOR* gene. Except yeast, which in some cases contains two different TOR genes, higher eukaryotes possess only a single copy of TOR (Wullschleger et al. 2006). The fact that TOR is a large protein containing multiple HEAT repeats suggests that it may function in a complex with other proteins. Indeed, it has been shown that TOR is present in two different complexes, TOR-complex 1 and 2 (TORC1 and TORC2 respectively), with distinct functions (Guertin et al. 2004). While TORC1 is sensitive to rapamycin treatment, TORC2 is not (see

more about TORC2 below)(Loewith and Hall 2004). In both complexes, TOR is associated with the protein GßL (Lst8 in yeast), which binds to the kinase domain of TOR and acts as a positive regulator of mammalian and yeast TOR signaling (Wullschleger et al. 2006). However, the precise role of GβL is not known. In TORC1, TOR and GβL are associated with a third binding partner, the 150kD protein raptor (KOG1 in yeast), which contains several HEAT repeats and seven WD40 domains (Wullschleger et al. 2006). Albeit it is known that raptor and TOR interact directly, the domains where this interaction takes place still remains elusive, which suggests multiple contact sites between these two proteins. As mentioned above, TORC1 is sensitive to rapamycin treatment. Although the exact mechanism is unknown, the FKBP12-rapamycin complex binds directly to TORC1 and thereby downregulates its activity (Wullschleger et al. 2006).

TOR is an integrator of a big variety of different signals that influence growth of a cell. Four major inputs have been implicated in TOR signaling: growth factors, nutrients, energy, and stress. TOR is one of the principal control elements in the regulation of growth and energy metabolism. Although it still remains elusive, how and on which level the different signals influence TOR activity, it seems that most of them affect TOR by regulating the TSC1/2 complex (Wullschleger et al. 2006). As mentioned above, the integration of the growth signals is likely to function via the PKB dependent phosphorylation of TSC2. Also the energy status of a cell is transmitted by the TSC1/2 complex. The energy "sensor" AMP-activated protein kinase (AMPK) is activated in response to low cellular energy (high AMP/ATP ratio). Activated AMPK directly phosphorylates TSC2 and thereby enhances its GAP activity, leading to the inhibition of TORC1 signaling (Inoki et al. 2003). Further, cellular stresses, such as hypoxia, downregulate TOR activity, and thereby inhibit protein synthesis (Wullschleger et al. 2006). Hypoxia is transduced to TORC1 via the two homologous proteins REDD1 and REDD2 (scylla and charybdis in *Drosophila*)(Brugarolas et al. 2004; Reiling and Hafen 2004). REDD, whose expression is induced by the hypoxia inducible factor-1 (HIF-1), acts downstream of PKB and upstream of the TSC1/2 complex to inhibit TORC1 signaling (Reiling and Hafen 2004). Finally, nutrients, especially amino acids, regulate TORC1 activity. Amino acid deprivation results in rapid dephosphorylation of TORC1 downstream targets. This dephosphorylation is strictly TORC1 dependent (Wullschleger et al. 2006).

To current knowledge, the TORC2 consists of TOR, GβL, and a third binding partner named rictor (also known as AVO3 from yeast) but not raptor (Jacinto et al. 2004; Sarbassov et al. 2004). Rictor is a large protein (~200kDa) that contains no obvious catalytic motifs. In contrast to raptor, knockdown of TOR and rictor results in both actin polymerization and cell

spreading (Jacinto et al. 2004; Sarbassov et al. 2004). Besides its role in cytoskeleton reorganization, TORC2 has been found to phosphorylate and activate PKB (see also above)(Sarbassov et al. 2005). Thus, TORC2 plays a positive part in the activation of PKB. Importantly, PKB is phosphorylated by TORC2 but not by TORC1. The biological implications of this phosphorylation of PKB by TORC2, especially the indirect control of TORC2 over TORC1, which is activated by PKB, remains elusive. Apart from the effects on the cytoskeleton and PKB phosphorylation, the function of TORC2 is unclear. It is important to note that, due to the recent finding of TORC2, the effects of most upstream signals on TOR have been studied only in the context of TORC1. It is even unknown whether TORC2 is similarly influenced by the upstream regulators TSC and Rheb.

Downstream of TOR

TOR signaling plays a role in various growth-related processes in yeast and higher eukaryotes. The best-studied targets of TOR are the translation regulators S6K and 4EBP. S6K is an InR/TOR downstream signaling component with a direct impact on the translational machinery. S6K is a Ser/Thr kinase that is activated upon PDK1- and TORC1-dependent Ser/Thr phosphorylation (Oldham and Hafen 2003). S6K in turn phosphorylates the ribosomal protein S6 of the small ribosomal subunit. Phosphorylated S6 increases the translation of a subclass of mRNAs containing a 5'-tract of oligopyrimidines (5'TOPs), including many ribosomal proteins, elongation factors, and the poly(A)-binding protein (Leevers and Hafen 2004). The 5'-TOP mRNAs account for 15-20% of total cellular mRNAs (Wullschleger et al. 2006). Thus, by regulating S6K activity, TOR increases the translational capacity of a cell by selectively increasing the pool of ribosomes and associated factors. However, this model has been recently challenged by experiments showing that translation of 5'TOP mRNAs does not depend on S6K activity nor on S6 phosphorylation (Pende et al. 2004; Ruvinsky et al. 2005). Thus, it remains to be tested how and if TORC1 controls 5'TOP mRNAs. Further, it should be noted that S6K has also other biological targets which can affect translation and cell growth, including the protein synthesis elongation factor 2 kinase (eEF2K)(Wang et al. 2001) and 4EBP (Raught et al. 2001). A second way in which TORC1 activation promotes translation is via the direct phosphorylation and inactivation of 4EBP (see also above).

Phosphorylated 4EBP no longer binds to eIF-4E, which is than free to associate with eIF-4G to stimulate translation initiation.

Apart from stimulating the onset of translation, it has been shown that TOR also controls ribosome biogenesis (Wullschleger et al. 2006). Studies in both yeast and mammalian cells have demonstrated that rapamycin blocks the biosynthesis of ribosomes by inhibiting transcription of RNA polymerase I (Pol I)-dependent rRNA genes, Pol II-dependent ribosomal protein genes, and Pol III-dependent tRNA genes (Martin and Hall 2005). Thus, TOR links nutrient availability to the biosynthesis of ribosomes, which accounts for a large segment of total energy consumption by the cell. However, the mechanism by which TOR controls the activity of all three RNA polymerases in a coordinated manner is not yet understood. In addition to the regulation of genes involved in ribosome biogenesis, TORC1 also controls the transcription of many other genes, particularly genes involved in metabolic and biosynthetic pathways. This has been demonstrated by microarray experiments on rapamycin-treated mammalian cells (Peng et al. 2002). Further, it has been shown that TORC1-mediated phosphorylation regulates the nuclear localization and the activity of several nutrient- and stress-responsive transcription factors in yeast (Loewith and Hall 2004). All in all, the role of TOR-controlled transcription in mediating cell growth remains poorly understood.

Another process that is regulated by TOR, but also by the PI3K pathway, depending on the nutritional supply, is autophagy. Autophagy is a process by which eukaryotic cells recover their own cytoplasmic contents, including organelles, under starvation conditions to recycle amino acids and other macromolecules to provide an internal reserve of nutrients. This catabolic process involves the enclosure of cytoplasm by a double-membrane structure (autophagosome) and its subsequent delivery to the lysosome (Wullschleger et al. 2006). While *TOR* mutant *Drosophila* larvae display a strong enhancement of autophagy in the fat body even under non-starvation conditions, TOR overexpression represses starvation-induced autophagy (Scott et al. 2004). Although TOR is an important regulator of autophagy, it is not the only one. The control of autophagy is nutritionally, hormonally, and developmentally regulated by multiple signaling pathways (Levine and Klionsky 2004; Meijer and Codogno 2004; Rusten et al. 2004).

Feedback loops between the InR and TOR pathway

In *Drosophila*, the activity of PKB is increased in *TOR* and *S6K* mutant larvae, and decreased in the absence of a functional TSC complex. These observations suggest that S6K inhibits PKB activity via a negative feedback loop (Radimerski et al. 2002; Lizcano et al. 2003). Although the existence of this negative regulation could be clearly shown, the molecular mechanism in insects is unknown. However, it has been also shown in earlier studies of mammalian tissues that amino acid excess inhibits insulin signaling in a rapamycin-dependent manner (Tremblay et al. 2005). Further it could be shown in mouse embryonic fibroblasts (MEFs) that loss of either TSC1 or TSC2 leads to a strong inhibition of insulin-mediated PI3K signaling (Manning 2004). Recently, it has been demonstrated that the negative feedback mechanism in vertebrates functions by direct phosphorylation and inactivation of IRS proteins. In particular, S6K1 regulates IRS1 both at the transcriptional level and through direct phosphorylation, thereby impairing IRS1 adaptor function. Further, S6K1 knockout mice are, due to the loss of the IRS inhibition, hypersensitive to insulin (Um et al. 2004). Thus, constitutive activation of TOR-S6K signaling induces a negative feedback loop to attenuate PI3K via inhibition of IRS. In this light, the rather benign nature of hamartomas caused by the loss of either *TSC* gene in comparison to the malignant *PTEN* mutant tumors might be explained by insufficient PI3K/PKB pathway activity in these tumor cells necessary for a malignant conversion.

Mutant phenotypes of the IIS pathway in **Drosophila**

The most obvious role of IIS, uncovered by genetic analysis in *Drosophila*, is the regulation of growth and body size without disturbing patterning. A characteristic of the growth differences caused by IIS alterations (at least the pathway components from the InR to PKB) is that both cell number and cell size are affected, which is reminiscent of flies reared under adverse nutritional conditions. While positive components of the pathway stimulate growth, the negative ones restrict it. However, strong mutations in the InR, PI3K, PTEN, and PKB are all recessive embryonic lethal, indicating an essential function of those proteins during normal development (Garofalo 2002). Viable combinations of hypomorphic alleles of InR, PI3K, and PKB lead to the development of small flies with reduced cell sizes and numbers, which show

female sterility, a developmental delay, and at least in the case of the InR increased longevity (Hafen 2004). The reduction in cell growth and cell number is strictly autonomous, and is neither affecting differentiation nor patterning. Loss-of-function mutations in the adaptor protein chico are semi-viable, but are also delayed, reduced in size, due to a decrease in cell size and number (Bohni et al. 1999), and live significantly longer (Clancy et al. 2001). The reason why Chico is the only non-essential protein of the canonical IIS cascade upstream of PKB, could be that the InR is able to directly signal to PI3K, thereby circumventing the IRS adaptor protein (Leevers and Hafen 2004).

In *Drosophila*, cell size and cell number appear to be controlled partly by two separate branches of the IIS network. While cell size but not cell number is reduced in mutants lacking S6K (Montagne et al. 1999), in *chico* mutants, only the reduction in cell number is depending on dFoxO (Junger et al. 2003). Intriguingly, although dFoxO is necessary for controlling the cell number-output of IIS, dFoxO mutants are viable and of normal body size when reared under standard culture conditions (Junger et al. 2003; Kramer et al. 2003).

The role of IIS in the female reproductive system can be divided into two different aspects of its function. First, IIS controls the development of the female reproductive system. Female flies containing a temperature-sensitive allele of *dp110* (*dp110*^{ts}) become sterile when reared close to the restrictive temperature during larval and pupal development, but are kept at the permissive temperature during their adult life (Dietz 2003). Thus, IIS is needed for the development of the ovaries, and not only for egg production itself. Second, *dp110*$^{ts/ts}$ females reared at the permissive temperature during their whole development display a normal egg laying behavior, but when shifted to the restrictive temperature after hatching arrest egg laying within four days (Dietz 2003). Shifting the flies back to the permissive temperature can reverse this arrest in oogenesis. This reversible block in oogenesis is similar to what can be observed in starved and re-fed females (Drummond-Barbosa and Spradling 2001). Thus, besides playing a role in the development of the ovaries, IIS presumably regulates egg production according to the availability of nutrients.

In addition to its effects on growth and reproduction in insects, the IIS pathway has also been shown to affect energy homeostasis: *chico* mutants have massively increased fat storages compared to wild type flies (Bohni et al. 1999), and PI3K loss-of-function leads to elevated glucose levels in the circulation of adult flies (M. Jünger and E. Hafen, unpublished results). Thus, not only the regulation of growth, but also the regulation of circulating glucose by insulin seems to be evolutionarily conserved between metazoans and vertebrates which develop severe hyperglycemia when lacking the InR (Louvi et al. 1997).

The similarity of the phenotypes caused by the loss of components in the insulin signaling pathway in insects and vertebrates strongly suggests a conserved role of this pathway in the control of overall growth during development without impinging on pattern formation. If, and to what extent also other functions of the vertebrate insulin family (e.g. ovarian development or energy metabolism) are conserved in *Drosophila* is still unclear.

The Drosophila endocrine system

Almost every aspect of an insect's life is regulated by hormones at various developmental timepoints. Although the best-studied endocrine-stimulated events in their life cycle are molting and metamorphosis, there is a variety of others, such as metabolism, water-balance, reproductive cycle, and diapause, as well as behaviors such as eclosion, pheromone production, and migration, which remain largely obscure (the following text about the endocrine system is referred to in (Nijhout 1994)).

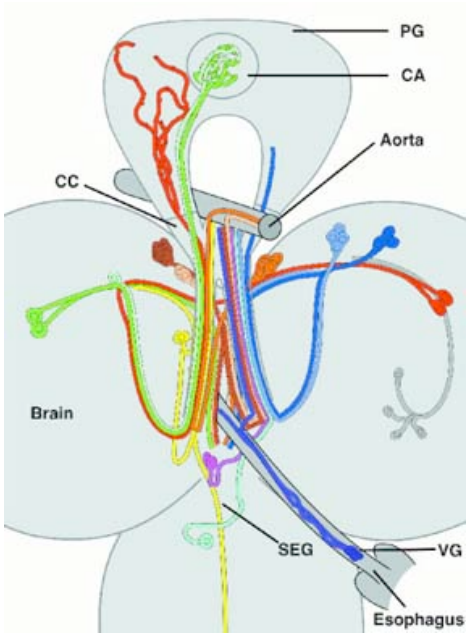


Figure 2: The Drosophila Brain-Ring Gland Complex
Structure of the Drosophila brain and ring gland and some of its connections (adapted from (Siegmund and Korge 2001)). Abbreviations: CA, corpora allata; CC, corpora cardiaca; PG, prothoracic gland; SEG, subesophageal ganglion; VG, ventricular ganglion.

Anatomy

The insect endocrine system, as well as the vertebrate system, is composed of two different endocrine organs: the conventional glandular tissues and the neurosecretory cells.

The two principal endocrine glands in insects are the prothoracic glands (PG), which produce and secrete ecdysteroids, and the corpora allata (CA), where juvenile hormone (JH) is produced and released. In higher dipterans such as *Drosophila*, the PG and the CA build together with the glandular/neurohemal (see below) organ corpora cardiaca (CC) a compound gland (King et al. 1966), which is located anterior to the brain hemispheres in the larva. Because of its circular shape, this compound gland is called ring gland (RG, Fig. 2). During metamorphosis, the PG (contrary to the CC and the CA) undergoes programmed cell death (Dai and Gilbert 1991) in all insects, except in certain *Apterygota* and the ovaries and the testes start to produce and release ecdysteroids.

The neurosecretory cells (NSC), the second kind of endocrine organ, consist of groups of specialized neurons in the central nervous system. NSC are abundant in the brain (but occur actually in all ganglia) and have unusually large cell bodies that, instead of neurotransmitters, produce small polypeptides, the neurohormones. NSC usually do not secrete their products at synaptic endings but send their axons to specialized structures where their secretions are released directly into the hemolymph. Such compact release sites are called neurohemal organs. The CC serves as the principal, though not the exclusive, neurohemal site for the NSC of the brain. The name CC is derived from the fact that in many species it is intimately associated with the heart (it is actually connected to the aorta in *Drosophila*), where it releases the secretions into the hemolymph. Besides common neurons, the CC contains a number of intrinsic NSC that produce and release their hormones locally, and thus compose the glandular part of the CC.

As in vertebrates, the secretory activity of the conventional endocrine glands in insects is controlled by the secretion of tropic or inhibitory neurohormones produced in the NSC. Thus, nearly every hormone that is not produced or secreted by NSC, is usually controlled by two neurohormones, a stimulating and an inhibiting one. Among other reasons, this is why the majority of insect hormones are such neurosecretory products.

The primary hormones

Among the first hormone families discovered were the ecdysteroids (EC). EC are steroid hormones occurring in both insects and plants. In insects and other arthropods more than sixty ecdysteroids have been isolated (Rees 1989), whereas more than hundred “phytoecdysteroids” are known in plants (Lafont and Horn 1989). Some of the phytoecdysteroids are identical

(have the same molecular structure and composition) to known insect EC, others are so far only known from plants. During larval development of *Drosophila*, as mentioned above, EC are the principal product of the large cells at both sides of the ring gland, called PG, whereas in adult flies the source of EC synthesis and release are the ovarian follicle cells and the gonads. The EC are not believed to be stored by the PG because the hormone is generally not detectable in homogenates of the glands even during periods of high activity. Thus, ecdysone, the hormone secreted by the PG and a member of the EC family, appears to be released as soon as it is synthesized. While ecdysone is relatively inactive and generally believed to be a prohormone, 20-hydroxyecdysone (20-HOE) is the active EC in most insects. 20-HOE is produced from ecdysone by the action of a P450 monooxygenase that hydroxylates E at carbon 20. In *Drosophila*, it has been shown that the gene *shade* is responsible for this transition from ecdysone to 20-HOE (Petryk et al. 2003). Interestingly, *shade* is not expressed in the ring gland itself, but in peripheral tissues such as epidermis, midgut, Malpighian Tubules and fat body (Petryk et al. 2003). Thus, although ecdysone is produced and secreted by the PG, the transformation into the active 20-HOE occurs at various other locations throughout the body. Because it is secreted prior to each molt, ecdysone is also called the "molting hormone". The direct action of 20-HOE on the epidermal cells causes them to undergo apolysis, cell division, digestion of the old cuticle, and secretion of a new cuticle. It has to be mentioned that ecdysone, like most of the ring gland hormones, is mainly controlled by the brain, namely through prothoracicotropic hormones (PTTH).

PTTH are neurosecretory polypeptides that are produced by lateral NSC in the anterior brain region and are transported via axons into the ring gland. Probably the axons release the PTTH directly in the PG, where they stimulate the synthesis and secretion of ecdysone. Thus, during larval development, every ecdysone peak is preceded by a PTTH peak. PTTH itself is negatively regulated by JH, but surprisingly only in the final larval instar of holometabolous insects (Nijhout and Williams 1974; Rountree and Bollenbacher 1986). What regulates PTTH in earlier instars is still unclear.

Another hormone that plays a major role in insect development and reproduction, is the JH. According to (Nijhout 1994), JHs are probably the most versatile hormones in the entire animal kingdom. They are involved in diverse processes such as metamorphosis, larval and adult diapause regulation, vitellogenin synthesis (by the fat body), longevity, ovarian development, and various aspects of metabolism. So far, the only known source of JH synthesis and release in insects is the CA, a small glandular tissue (see above) that is innervated by several axons from the brain and probably the CC. The CA does not store JHs,

so that the rate at which JH secretion occurs is determined entirely by the rate of its synthesis. The synthesis itself is largely dependent on neuropeptides from the brain. The production of JH from the CA is stimulated by allatotropic hormones (ATH, Atn in *Drosophila*) and inhibited by allatostatic hormones (ASH, Ast in *Drosophila*). Another neuropeptide that has just recently been shown in *Drosophila* to positively regulate JH production is insulin (Tatar et al. 2001). JHs are sequiterpenoids, derived from farnesenic acid, with an epoxide group near one end and a methyl ester on the other (Röller et al. 1967). The natural JHs are extremely unstable and get degraded by sunlight and various esterases in the hemolymph. JH is a lipidlike molecule that has two mechanisms of action at the cellular level: in the fashion of a steroid hormone, binding to high affinity receptors in the nucleus, and in the manner of a peptide hormone, binding to a cell surface receptor activating a phosphatidyl inositol-mediated second-messenger system. Although a lot about the distinct functions of JH is still unclear, it is known that high JH levels prevent metamorphosis and adult differentiation, whereas the degree of its absence determines whether the animal molts to a pupal, larval or adult form (Harvie et al. 1998).

Akh - another insect hormone involved in controlling metabolism

The adipokinetic hormones (AKH) form another important hormone class in insects, of which some members play a crucial role in metabolism. The structure of the AKHs is very similar to that of the crustacean red-pigment-concentrating hormone (RPCH) family, which regulates the expansion of pigment in red chromatophores. Because of this structural relationship, the family is called the AKH/RPCH family. It forms a widespread and functionally diverse family of small neuropeptides, of which hypertrehalosemic hormone (HTH) is a member in insects. HTH was first discovered in CC homogenates of the cockroach *Periplaneta americana* (Steele 1961) as a factor that causes, when injected, a dramatic elevation of trehalose (a disaccharide of glucose and the main form of carbohydrate in the hemolymph of most insects) in the hemolymph and cardioacceleration. It actually stimulates the breakdown of glycogen via activation of the enzyme phosphorylase, which is the rate-limiting enzyme in the breakdown pathway of glycogen (Mordue and Goldsworthy 1969; Goldsworthy 1970; Ziegler 1979; Gäde 1981; Steele 1982; Steele 1985). HTHs are neurosecretory hormones that usually contain 8-10 amino acids (aa) and are produced, in all the tested insect species, by the intrinsic neurosecretory cells of the CC. HTH, as all the AKHs, does not appear to have

species-specific functions (e.g. AKH from the blowfly *Phormia* is recognized by receptors in the fat body of the cockroach *Periplaneta*, which leads to an elevation of carbohydrate in the hemolymph). Considering the function of HTH, it has to be mentioned that there exist a slight sequence similarity between AKHs and the N-terminal portion of glucagon (Scarborough et al. 1984). Given the fact that all the members of the AKH family show high structural similarity, it is not quite clear what accounts for the differences of their biological functions. Much of the diverse regulatory functions may be due to differences in the receptor and the transduction mechanisms of the target cells.

In *Drosophila*, a protein with high homology to the AKH/RPCH family, and thus also to HTH, has been discovered: the *Drosophila* adipokinetic hormone (*dAkh*)(Schaffer et al. 1990). The dAkh is produced as a 79 amino acid precursor protein, which is processed into an octapeptide with, like most members of the AKH family, a pyroglutamic acid at its N-terminus and a tryptophan carboxyamide at its C-terminus. Thus the N- as well as the C-terminus is blocked. dAkh also contains the characteristic phenylalanine residue at position 4 and the tryptophan residue at position 8. An unusual feature about the sequence of dAkh is, that it contains a charged residue, i.e. aspartic acid, at position 7. All the other members of the AKH/RPCH family known so far are uncharged, with the exception of a recently discovered protein of the blowfly *Phormia terraenovae* (Diptera), called *Phormia terraenovae* hypertrehalosemic hormone (Pht-HrTH)(Gäde et al. 1990). Pht-HrTH is in sequence and structure absolutely identical to the dAkh. It has been shown that dAkh elevates the carbohydrate level in the blood in a dose-dependent manner (without an increase of hemolymph lipid levels) and accelerates the heartbeat when injected into the blowfly *Phormia terraenovae* and the cockroach *Periplaneta americana* (Gäde et al. 1990). Additionally, it has been shown in *Drosophila* that the decrease in trehalose levels following targeted ablation of the dAkh producing CC cells is partly rescued by expression of a dAkh transgene (Kim and Rulifson 2004). Besides its hyperglycemic activity, dAkh also displays cardioaccelerating activity in *Drosophila* prepupae (Noyes et al. 1995). Thus, dAkh is a functional member of the HTH superfamily, which holds the ability to increase hemolymph trehalose content and to accelerate the heartbeat.

Results

The results part is composed of two different parts:

- **The manuscript of "Imp-L2 binds Dilp2 and counteracts insulin signaling in *Drosophila melanogaster*"**

- **Supplemental results about Imp-L2**

Imp-L2 binds Dilp2 and counteracts insulin signaling in Drosophila melanogaster (Manuscript)

Basil Honegger[1], Milos Galic[1], Katja Köhler[1], Franz Wittwer[1], Walter Brogiolo[1], Ernst Hafen[2,3]

[1] Zoologisches Institut
Universität Zürich
Winterthurerstr. 190
CH-8057 Zürich
Switzerland

[2] Institute for Molecular Systems Biology (IMSB)
ETH Zürich
Wolfgang-Pauli-Str. 16
CH-8093 Zürich
Switzerland

[3] corresponding author (ernst.hafen@sl.ethz.ch)

Abstract

The highly conserved insulin/IGF receptor pathway plays a predominant role in determining size, metabolism, reproduction, and longevity. Here we show that a secreted member of the immunoglobulin superfamily, *Imaginal morphogenesis protein-Late 2* (*Imp-L2*), which is homologous to the potential vertebrate tumor suppressor IGF binding protein-related protein 1 (IGFBP-rP1), inhibits growth non-autonomously by suppressing insulin signaling in *Drosophila*. *Imp-L2* is expressed in parts of the major endocrine tissue, the corpora cardiaca, a variety of brain neurons, and the same seven median neurosecretory cells that produce four of the seven *Drosophila* insulin-like peptides (Dilps). While overexpressing *Imp-L2* reduces size dramatically, loss-of *Imp-L2* function increase overall body size to the same extent as weak *dilp2* overexpression. An increase of Dilp2 levels in an *Imp-L2* mutant background results in lethality. Thus, *Imp-L2* is necessary to compensate hyperinsulinemia in *Drosophila*. The fact that co-overexpression of *Imp-L2* rescues the lethality induced by high Dilp2 levels, and that Imp-L2 binds Dilp2 *in vitro*, strongly suggests that Imp-L2 plays a predominant role in regulating Dilp2 activity *in vivo*. Furthermore, we show that under starvation conditions, *Imp-L2* is necessary for larval survival and for efficiently inhibiting insulin signaling.

Introduction

In higher organisms, growth is regulated by a variety of intrinsic and extrinsic factors throughout development. Dysfunction of the signaling pathways controlling growth results in cells of altered size and contributes to a wide variety of pathological conditions, such as cancer, diabetes, and inflammation (reference). One of the major pathways controlling growth is the evolutionarily conserved insulin/IGF receptor (*InR*/*IGFR*) pathway. In mammals, the primary role of insulin and the InR is energy homeostasis by regulating blood glucose levels (Saltiel and Kahn 2001), but mutations in the *InR* gene can also cause embryonic growth retardation (Taylor 1992; Takahashi et al. 1997). However, the main growth regulatory function in mammals is carried out by the insulin-like growth factors (IGF)-1 and -2 and the IGFR (Butler and Le Roith 2001).

Drosophila has a single InR (dInR) that acts via a canonical phosphatidylinositide 3-kinase (PI3K)/protein kinase B (dPKB/dAkt) pathway which regulates growth, metabolism, reproduction, and longevity (Garofalo 2002). Upon activation, InR phosphorylates adaptor

proteins like insulin receptor substrates (IRS1-4, the only homolog in *Drosophila* is called *chico*), thereby creating a binding site for the SH2-domain containing regulatory subunit of class IA PI3K, p85 (dp60 in *D.m.*). Via its catalytic p110 (dp110 in *D.m.*) subunit, PI3K phosphorylates phosphatidylinositol-(4,5)-bisphosphate (PIP_2) to elevate phosphatidylinositol-(3,4,5)-trisphosphate (PIP_3) levels. This phosphorylation is negatively regulated by the tumor suppressor PTEN (phosphatase and tensin homolog on chromosome ten). PIP_3 serves as binding site for the PH-domain of PKB, recruiting it to the cell membrane, where it is phosphorylated by the phosphoinositide-dependent kinase 1 (PDK1) and the target of rapamycin complex 2 (TORC2) (Sarbassov et al. 2005). Among others, activated PKB phosphorylates the transcription factor FoxO (forkhead box, subgroup "O") thereby inhibiting its function.

Recently, seven insulin-like peptides (Dilps) serving as putative ligands for the insulin receptor have been identified in *Drosophila* (Brogiolo et al. 2001). *Dilps* are expressed in a highly controlled spatial and temporal pattern, which includes expression in median neurosecretory cells (m-NSCs) of both brain hemispheres. The m-NSCs possess axon terminals in the larval endocrine gland and on the aorta, where the Dilps are secreted into the hemolymph and act in an endocrine fashion to control growth (Brogiolo et al. 2001; Ikeya et al. 2002; Rulifson et al. 2002). Ablation of these m-NSCs causes a developmental delay, growth retardation, and elevated carbohydrate levels in the larval hemolymph (Ikeya et al. 2002; Rulifson et al. 2002). These defects mimic the phenotype of starved flies or flies mutant for insulin signaling components, and can be rescued by ubiquitous expression of *dilp2* (Rulifson et al. 2002).

Overexpression of each of the seven *dilps* is sufficient to increase total body size (Ikeya et al. 2002). Apart from their effect on size, nothing is known about the specific function of the different Dilps. Since no loss-of-function mutations exist in any of the *dilp* genes, it is unknown if the different Dilps carry out distinct functions or if their function is similar but exerted on different target tissues. In biological fluids of vertebrates, IGFs are normally bound and transported by IGF binding proteins (IGFBP) (Hwa et al. 1999). Having higher affinity for IGFs than for the IGFR, IGFBPs not only prolong the half-lives of IGFs, but modulate their availability and activity (Jones and Clemmons 1995). Besides the classical IGFBP, a related protein named IGFBP-related protein-1 (IGFBP-rP1/IGFBP-7/mac25/TAF/AGM/PSF) has been identified as a high-affinity insulin binding protein (Yamanaka et al. 1997). Thus, extracellular insulin/IGF signaling in vertebrates is controlled

by a family of binding proteins that modulates interactions of the ligand with the receptor. In *Drosophila* nothing is known about the control of extracellular Dilp levels.
In a gain-of-function screen for suppressors of *dInR*-induced hyperplasia in the eye, we identified the secreted protein Imaginal morphogenesis protein-Late2 (Imp-L2) that displays some homology to IGFBP-rP1. While overexpression of *Imp-L2* results in a non-autonomous size reduction, loss-of *Imp-L2* function enhances insulin signaling and increases organismal size. Since Imp-L2 binds Dilp2 *in vitro* and antagonizes its function *in vivo*, we propose that Imp-L2 acts as an insulin binding protein in *Drosophila* necessary to block insulin signaling under adverse nutritional conditions.

Results

Identification of Imp-L2

To identify novel inhibitors of the Insulin signaling pathway, we performed a misexpression screen using a double-headed enhancer-promotor (EP) element. We tested 5400 random EP insertions for suppression of the overgrowth phenotype caused by the overexpression of wild-type *dInR* in the developing eye using GMR-*Gal4* (Fig. 1A). It has already been demonstrated that this system is sensitive to alterations in insulin signaling both up- (Brogiolo et al. 2001) and downstream (Wittwer et al. 2005) of the *dInR*. Thus, our screening system is likely to identify further components of the pathway.
Among the suppressor lines identified, one contained an EP element (EP5.66) inserted 8.5kb upstream of the *Imp-L2* coding sequence (Fig. 1F). When driven by GMR-*Gal4*, EP5.66 strongly suppressed the *dInR* induced big-eye phenotype (Fig 1B). The *Imp-L2* gene locus (Fig. 1F) consists of three different transcripts that differ in the usage of the first exon but share the same *Imp-L2* protein-coding region. While *Imp-L2-RA* and *Imp-L2-RC* contain a small non-coding first exon (Fig. 1F), *Imp-L2-RB* possesses three small upstream open reading frames (uORFs) in its first exon. We established two different UAS transgenes containing the cDNA of the *Imp-L2-RB* transcript either with (UAS-*long-IMPL2*, further named UAS-*l.IMPL2*) or without (UAS-*short-IMPL2*, further named UAS-*s.IMPL2*) the first exon. While UAS-*l.IMPL2* only partially suppresses the InR induced bulging eye phenotype (Fig. 1D), UAS-*s.IMPL2* entirely reversed the phenotype (Fig. 1E). Additionally, a point

mutation in the Imp-L2 coding sequence (see below) abolishes its ability to suppress the InR caused overproliferation (Fig. 1C). Thus, Imp-L2 is sufficient to suppress the dInR induced eye phenotype. Furthermore, these results indicate that the first exon of the *Imp-L2-RB* transcript has a repressive function on the translation of the *Imp-L2* mRNA (see also below). *Imp-L2* has been previously shown to be upregulated 8-10 hours after ecdysone treatment (Osterbur et al. 1988) in a screen for ecdysone-inducible genes (Natzle et al. 1986). It represents a secreted member of the immunoglobulin (Ig) superfamily containing two Ig-C2-like domains. Although there are several orthologs of Imp-L2 in lower organisms as arthropods and nematodes (Fig. 1G), no clear structural homolog could be identified in higher organisms.

In vertebrates, insulin and IGFs are part of a tightly controlled network of ligands, receptors and binding proteins (for a review see (Hwa et al. 1999)). While the classical IGFBP-1 to -6 have very high affinities for IGFs, their binding affinity for insulin is very low. On the other hand, IGFBP-rP1 binds insulin, IGF-I and -II to about the same extent, but its insulin:IGF binding ratio is 500-fold higher than that of IGFBP-1 to -6 (Yamanaka et al. 1997). Structurally, the IGFBP1-6 and IGFBP-rP1 show a high degree of similarity in their N-terminal domain, whereas the C-terminal ends differ considerably. Instead of the classical IGFBP C-terminus IGFBP-rP1 contains an Ig-C2 like domain, which shows, especially around the cysteins forming the disulfide bridges, similarity to the second Ig domain of Imp-L2 (Fig. 1G). Additionally, it has been shown by Sloth Andersen and colleagues that Imp-L2 is also able to bind human insulin, IGF-I, and IGF-II (Sloth Andersen et al. 2000). Thus, it is likely that Imp-L2 is the functional homolog of IGFBP-rP1 in lower organisms which lack other IGFBP orthologs.

The expression of Imp-L2 is highly regulated during development

As shown by Garbe and colleagues, *Imp-L2* mRNA can be detected in embryos from the cellular blastoderm throughout embryogenesis in a variety of different tissues, including the ventral neuroectoderm, the tracheal pits, the pharynx, the esophagus, and specific neuronal cell bodies (Garbe et al. 1993). Nevertheless Imp-L2 protein is only localized to specific neuronal structures late in embryogenesis (Garbe et al. 1993). To further determine the expression pattern of Imp-L2, we performed *in situ* hybridization and immunohistochemistry in larval and adult tissues. In 3rd instar larvae, *Imp-L2* mRNA and protein are strongly

expressed in the corpora cardiaca (CC, Fig. 2A, D, E), which are part of the ring gland. The ring gland is the major neuroendocrine organ of *Drosophila* larvae, controlling metabolism, development and growth. In addition to the staining in the CC, weaker expression of Imp-L2 can be detected in several neurons of both brain hemispheres, the subesophageal ganglion (SOG) and the ventral ganglion (Fig. 2B and E). The seven m-NSC that produce the Dilps 1, 2, 3 and 5 (Fig. 2G), and project their axons directly to the SOG, the CC and the heart (Cao and Brown 2001; Rulifson et al. 2002) also express Imp-L2. Thus, Imp-L2 is expressed in the same cells as the *Drosophila* insulins, thereby having the potential to interact with the Dilps directly at their origin. Furthermore, Imp-L2 expression can be detected in 10-20 cells of the anterior midgut epithelium (Fig. 2C+F). These cells are much smaller than the surrounding cells (Fig. 2C'), and neither their origin nor their function is known.
In adults, Imp-L2 protein is expressed in distinct neurons of both male and female brains (Fig. 2I), and early stages of ovary development (Fig. 2H). In oogenesis, Imp-L2 is expressed from the germarium until stage 3 oocytes. After stage 3, the dot-like structures containing Imp-L2 protein disappear gradually. Beyond stage 6, no more Imp-L2 protein can be detected. Contrary to the uniform cytoplasmic staining observed in other tissues, Imp-L2 is expressed in vesicle-like compartments in the ovary (Fig. 2H).
In summary, Imp-L2 is expressed in a variety of neuronal and secretory tissues. Amongst them are cells controlling food uptake (Melcher and Pankratz 2005), glucose homeostasis, energy metabolism (Ikeya et al. 2002; Rulifson et al. 2002; Kim and Rulifson 2004), and reproduction. This is further supporting the hypothesis that Imp-L2 is acting as a neuronally controlled hormone playing a role in regulating energy balance.

Imp-L2 reduces growth non-autonomously by reducing insulin signaling

To further assess the function of Imp-L2 as a secreted inhibitor of insulin signaling, we ectopically expressed *Imp-L2* by various Gal4 drivers in different tissues. Strong ubiquitous overexpression of *Imp-L2* by Act-*Gal4* led to lethality with both the UAS-*l.IMPL2* and the UAS-*s.IMPL2* transgenes. While driving UAS-*s.IMPL2* by the weaker arm-*Gal4* driver still resulted in lethality, driving UAS-*l.IMPL2* generated flies that were decreased in size and weight (-15% in males and -29% in females, data not shown). Apart from the size reduction, these flies eclosed with Mendelian ratio and had wild-type appearance. By generating overexpression flp-out clones in the eye we confirmed that cell specification and patterning of

Imp-L2 overexpressing ommatidia were normal (Fig. 3A). However, a reduction of cell size in the clones was observed. The reduction in cell size appeared to be non-autonomous since wild-type ommatidia close to the clone were also reduced in size. Given the convex nature of the eye we were unable to quantify effects of *Imp-L2* overexpression on more distantly located ommatidia. Thus, to assess the non-autonomous effect, total body size was measured, while *Imp-L2* was overexpressed in a tissue specific manner. Eye-specific overexpression of both UAS-*l.Imp-L2* and UAS-*s.Imp-L2* by GMR-*Gal4* led to a strong reduction in eye size (data not shown). While the GMR-*Gal4*, UAS-*l.Imp-L2* flies were of normal size, total body weight was reduced by 38.3% in GMR-*Gal4*, UAS-*s.Imp-L2* male flies (Fig. 3B), and development was delayed by one day. Because the highly specialized tissue of the *Drosophila* eye imaginal disc is unlikely to secrete sufficient Imp-L2 levels in order to elicit discernible effects on the rest of the body, we wanted to assay the effect of high Imp-L2 levels on total body size in a tissue better suited for protein secretion. Therefore, we used the ppl-*Gal4* driver to overexpress *Imp-L2* in the fat body. Ppl-*Gal4*, UAS-*s.IMPL2* flies were lethal, whereas ppl-*Gal4*, UAS-*l.IMPL2* flies showed a dramatic reduction in overall body size (Fig. 3C and D) and were developmentally delayed by two days. Both the size decrease and the developmental delay are reminiscent of phenotypes observed in mutants for positive components of the insulin signaling pathway such as *chico* (Bohni et al. 1999), further supporting the hypothesis that Imp-L2 acts as a negative regulator of this pathway.

Another characteristic of insulin signaling pathway mutations and nutritional limitation is a change in both cell size and cell number. Overexpressing UAS-*l.IMPL2* in the wing using C10-*Gal4* led to flies with smaller wings (Fig. 3E), which was mainly due to a reduction in cell number. The stronger UAS-*s.IMPL2* transgene again led to lethality when combined with the C10-*Gal4* driver. Therefore, we wanted to further investigate the inhibitory role of the first exon of the *Imp-L2-RB* transcript. For this purpose we generated UAS transgenes of both the *Imp-L2-RA* and the *Imp-L2-RC* transcripts, which lack any uORFs. Both UAS-*IMPL2-RA* and UAS-*IMPL2-RC* overexpressed in the wing decreased the wing size and cell number to a greater extent than UAS-*l.IMPL2* (Fig. 3E). In addition they also decreased cell size. Thus, overexpression of *Imp-L2* decreases body and organ size due to a reduction in both cell size and cell number.

To test whether the observed size reduction by *Imp-L2* overexpression is accompanied by a down-regulation of insulin signaling, we assayed its effect on PIP_3 levels. We used a GFP-PH domain fusion protein (tGPH) that specifically binds PIP_3 and serves as a reporter for PIP_3 levels *in vivo* (Britton et al. 2002). The amount of membrane bound tGPH reflects PI3K/PKB

signaling activity. Overexpression of *dInR* posterior to the morphogenetic furrow resulted in a severe increase of membrane PIP_3 levels (Fig. 4A and B). Like dPTEN, co-overexpression of *Imp-L2* together with *dInR* reduced PIP_3 levels significantly (Fig. 4C and D). This confirms that Imp-L2 inhibits PI3K/PKB signaling upstream of PIP_3, without affecting dInR levels (Fig. 4B' and 4D').

To further investigate the function of *Imp-L2*, we generated loss-of-function mutations in the gene. First we performed an EMS mutagenesis screen in which we selected mutated chromosomes carrying EP5.66 that were no longer able to suppress the *dInR* overexpression phenotype in the eye (Fig. 1A). Thereby, one revertant allele ($Imp\text{-}L2^{MG2}$) was identified that contained a point mutation in its ORF leading to a premature stop at amino acid 232 (Fig. 1F). The mutation is close to the C-terminal end of the protein and leads to a truncated version of Imp-L2 in which the conserved cysteine bridge of the 2nd Ig domain is destroyed (marked by an asterisk in Fig. 1G). Overexpression of this truncated *Imp-L2* version had no inhibitory effect on size (Fig. 1C). Thus, $Imp\text{-}L2^{MG2}$ is likely to be a functional null allele.
In addition, we generated further *Imp-L2* alleles by imprecise excision of GE24013, a P-element located 102bps upstream of the transcriptional start site of *Imp-L2-RC*'s first exon (Fig. 1F). We obtained four *Imp-L2* deficiencies lacking most (Def35) or the entire (Def20, Def42, Def223) coding sequence. All heteroallelic combinations of the mutant alleles increased total body size (Fig. 5A) and displayed enhanced insulin signaling (Fig. 7A compared with 7C). While homozygous mutant males showed a 27% increase of total body weight, mutant females were 64% heavier (Fig. 5B). Introducing one copy of a genomic rescue construct (Garbe et al. 1993) into homozygous mutant flies reverted the size to the level of $Imp\text{-}L2^{+/-}$ flies, which were already heavier (+14% in males, +44% in females, Fig. 5B). By measuring the cell density in the wing, the increase in size and weight could be attributed primarily to higher cell number, since cell size was only slightly affected (Fig. 5C). Apart from the size increase, the flies lacking *Imp-L2* appeared completely normal, eclosed with the expected frequency and were not delayed. Thus, *Imp-L2* loss-of-function dominantly increases growth by augmenting cell number without perturbing patterning, developmental timing or viability.
Interestingly, the weight difference in mutant females was about two-fold higher than in males (Fig. 5B), albeit the increase in wing area and cell number was similar. This is because *Imp-L2* mutant females had much larger abdomen than control flies (Fig. 5A). This phenotype was sensitive to crowding conditions within the vial, and not completely penetrant, which

suggests that Imp-L2 plays a role in the control of oogenesis. Although the mutant females (virgins or mated females) did not lay more eggs than controls (data not shown), ovaries of heterozygous and homozygous *Imp-L2* mutants were dramatically increased in size, similar to viable *dilp2* overexpression situations (Fig. 5D and not shown). Size and structure of the fly ovary are dependent on nutrition. The ovaries of females raised on poor food are reduced in size, and their ovarioles contain few, if any, vitellogenic stages (Drummond-Barbosa and Spradling 2001). This phenotype can be reproduced by inhibiting insulin signaling, since viable mutations in either the *dInR*, *chico*, *dPI3K*, *dPKB* or *dS6K*, cause female sterility (Chen et al. 1996; Bohni et al. 1999; Montagne et al. 1999) by preventing ovarian stages beyond the onset of vitellogenesis. Thus, there exists a checkpoint at the beginning of vitellogenesis that is sensitive to nutrition and under the control of the insulin signaling pathway. In *Imp-L2* mutant females, although the number of ovarioles is not changed, the amount of mature eggs per ovary is increased two-fold (Fig. 5E) and the number of vitellogenic stage10 egg chambers increased three-fold (Fig. 5E). Thus, *Imp-L2* is necessary for the checkpoint controlling the onset of vitellogenesis, without affecting the egg laying behavior.

Imp-L2 interacts with Dilp2 and is necessary for blocking insulin signaling under starvation

The fact that Imp-L2 is a secreted protein, and that removal of Imp-L2 function does not rescue either *chico* or *PI3K* mutant phenotypes (data not shown), is consistent with the hypothesis that it acts upstream of the intracellular insulin signaling pathway at the level of the ligands. Therefore, we wanted to test genetic interactions of *Imp-L2* with the *dilps*. A deficiency (*Df(3L)AC1*) uncovering *dilp1-5* dominantly enhanced the small eye phenotype caused by eye-specific overexpression of *Imp-L2* (supplementary info). *Dilp2* is the most potent growth regulator of the seven Dilps in *Drosophila* (Ikeya et al. 2002). Since flies lacking *Imp-L2* also display an increase in total body size, we further concentrated on the interaction between Imp-L2 and Dilp2. Weak ubiquitous overexpression of *dilp2* by arm-*Gal4* caused an increase in total body and organ size (Ikeya et al. 2002). Heterozygosity for *Imp-L2* dominantly increased the size of arm-*Gal4*, UAS-*dilp2* flies (Fig. 6A). Further, in homozygous *Imp-L2* mutants, expression of *dilp2* under the control of *arm*-Gal4 caused lethality, which is reminiscent of flies expressing *dilp2* at high levels (Ikeya et al. 2002). Expressing both *Imp-L2* and *dilp2* individually at high levels in the fat body also caused

lethality, but co-expression resulted in viable flies of wild-type size (Fig. 6B). Thus, *Imp-L2* decreases the sensitivity to high insulin levels and is sufficient to rescue the lethality resulting from *dilp2*-induced hyperinsulinemia. This raised the question whether Imp-L2 is able to physically bind Dilp2. Therefore, we constructed a tagged version of Dilp2 that contains an N-terminal Flag tag in between the signal peptide and the actual Dilp2 protein. Overexpression of *Flag-dilp2* in flies resulted in an increase of total body size demonstrating the functionality of the construct (not shown or supplementary info). Using *in vitro* translated, ^{35}S -labeled Imp-L2 and Flag-Dilp2 extracted from stably transfected S2 cells, we could show that Imp-L2 binds Dilp2 *in vitro* (Fig. 6C). Thus, by binding to Dilp2 and negatively regulating its function, Imp-L2 acts as a functional homolog of IGFBPs in *Drosophila*.

Imp-L2 is a potent inhibitor of Dilp2 action, but is not necessary for survival under standard conditions. Therefore we wanted to test whether Imp-L2 functions as a fast acting inhibitor of insulin signaling under stress conditions. We exposed wild-type and *Imp-L2* mutant early 3rd instar larvae to various starvation conditions and scored for survival. Larvae lacking *Imp-L2* showed a massive increase in lethality when exposed to 1% Glucose or PBS for 24h (Fig. 6D). To test whether the increased lethality of larvae under starvation was due to an inability to adjust insulin signaling, we monitored PIP3 levels under starvation conditions. Whereas control flies showed a decrease of PIP3 levels when exposed to complete starvation for 4h (Fig. 7B compared with 7A), *Imp-L2* mutant larvae still contained PIP3 levels that were comparable to control larvae reared on normal food (Fig. 7D compared with 7A), suggesting that Imp-L2 is necessary to shut down insulin signaling under starvation conditions. Imp-L2 could exert its insulin inhibiting effect under starvation either by enhancing the secretion of stored protein or by upregulating Imp-L2 levels directly. It has been shown in a microarray experiment by Zinke and colleagues that *Imp-L2* mRNA is weakly upregulated after exposure to 12h complete starvation (Zinke et al. 2002). We could not detect a change in expression of Imp-L2 in the brain, the ring gland and the gut after complete starvation for 24h (data not shown). However, Imp-L2 was upregulated in vesicular-like structures in fat body cells (Fig. 7E).

Taken together, these experiments show that Imp-L2 is upregulated by the fat body under adverse nutritional conditions, and that, although not crucial for survival on standard conditions, Imp-L2 is necessary for larval survival and blocking insulin signaling upon starvation.

Discussion

Imp-L2 modulates growth by regulating the insulin signaling pathway

A novel regulator of the insulin signaling pathway has been identified in a screen for suppressors of the InR induced hyperplasia in the eye. Imp-L2 regulates growth non-autonomously by downregulating the insulin signaling pathway activity *in vivo*. Depending on the levels, *Imp-L2* overexpression reduces body and organ size to the same extent and in a similar fashion as loss-of-function mutations in positive *dInR* signaling regulators, such as *chico* or *dPKB*. Like the negative regulator *dPTEN*, *Imp-L2* attenuates PIP3 levels induced by increased *dInR* activity, and releases PKB-dependent inhibition of dFoxO, thus enabling the expression of the dFoxO target gene *d4EBP* (not shown or refer to Franz screen paper). Loss-of *Imp-L2* function is sufficient to increase total body size to an extent that is similar to weak *dilp2* overexpression situations or viable PTEN loss-of-function alleles. Furthermore, in *Imp-L2* mutants, *dInR* signaling activity is increased and can no longer be attenuated under adverse nutritional conditions. The size increase of both *Imp-L2* mutants and viable *dilp2* overexpression is suppressed by removing *chico* (data not shown) suggesting that Imp-L2, like Dilp2, acts upstream of the intracellular insulin signaling pathway. Additionally, by genetic interactions and binding studies we showed that Imp-L2 binds Dilp2 directly and counteracts its functions. Therefore, we propose that Imp-L2 acts as a modulator of Dilp activity and its availability in biological fluids of insects.

Recently it has been shown that ecdysone, apart from its role as one of the major hormones regulating development and metamorphosis, antagonizes insulin signaling in determining final body size (Colombani et al. 2005). The larval fat body serves as a key relay element for this ecdysone-dependent growth inhibition (Colombani et al. 2005). Since Imp-L2 has been originally identified as an ecdysone-inducible protein and it is induced in the fat body upon starvation, it is tempting to speculate that the humoral, insulin-inhibiting signal emanating from the fat body could be the Imp-L2 protein. Further analysis of the growth inhibiting effect of ecdysone in an *Imp-L2* mutant background should enlighten this aspect of Imp-L2 function.

Is Imp-L2 the insulin binding protein used by lower organisms?

In vertebrates, IGF-1 and -2 are potent growth inducers that are mostly bound and regulated by their binding proteins (for review see (Hwa et al. 1999)). When bound to IGFs, IGFBPs function by regulating their transport between intra- and extravascular spaces, by interaction with their receptors (Zapf 1995), by prolonging their half-life (Stewart and Rotwein 1996) and by precluding their mitogenic activity (Rajah et al. 1999). In the vascular system, most of the binary IGF/IGFBP complexes are additionally bound to a third binding partner called acid labile subunit (ALS), whereby the half-life of the complex is again massively extended (Boisclair et al. 2001). While the affinity of IGFBP1-6 for IGF is very high, their ability to bind insulin is low. Conversely, IGFBP-rP1 binds IGF specifically with low affinity, but is a high-affinity insulin binding protein (Yamanaka et al. 1997). IGFBP-rP1 blocks binding of insulin to the InR and thereby inhibits the earliest step in insulin action (Yamanaka et al. 1997). Additionally, IGFBP-rP1 is a potential tumor suppressor (Burger et al. 1998) and plays an important role in the female reproductive system, in processes like endometrial receptivity, folliculogenesis as well as growth development and regression of the *corpus luteum* (Wandji et al. 2000; Dominguez et al. 2003; Casey et al. 2004).

Lower organisms like insects or nematodes, although lacking any clear orthologs of IGFBPs, produce various insulin-like proteins. The only known protein that has been implicated in insulin binding in invertebrates so far is Imp-L2. There is a single ortholog of Imp-L2 present in *Caenorhabditis elegans*, *Apis mellifera*, *Anopheles gambiae*, *Spodoptera frugiperda*, and *Drosophila pseudobscura*. In vertebrates, only homology between Imp-L2 and the Ig-domain containing C-terminus of IGFBP-rP1 could be detected. Although this is not very high, their function seems to be conserved. Like IGFBP-rP1, Imp-L2 is potently inhibiting growth, binds to and interacts with insulin, and plays a role in female reproduction in *Drosophila*. Therefore, we propose that Imp-L2 acts as the functional homolog of IGFBP-rP1 in *Drosophila*.

It is tempting to speculate that the IGFBPs in vertebrates evolved from the Imp-L2-like proteins, since in all lower organisms a single ortholog of these two-Ig-domain proteins seems to be present, but the characteristic N-terminal IGFBP-motif is missing completely. Thus, IGFBP-rP1 could be a chimerical protein that during evolution exchanged its N-terminal Ig-domain with a newly evolved IGFBP motif.

Recently, an ortholog of the glycoprotein ALS was also identified in *Drosophila* (*dALS*) (Colombani et al. 2003). *dALS* is expressed in the fat body and the *dilp*-producing m-NSCs, and, like in mammals, its expression is sensitive to starvation (Colombani et al. 2003).

However, the function of ALS in *Drosophila* is unknown. Interaction studies of dALS with the Dilps and Imp-L2 should clarify whether dALS is part of the network controlling the activity of insulin in flies.

Imp-L2 and Dilp2 interaction under starvation

In *Drosophila*, dInR/PI3K signaling coordinates nutritional status with growth and cell metabolism of endoreplicative tissues (ERT) like the fat body. Ectopic induction of *dInR* or *PI3K* is sufficient to drive cell growth in ERTs even in starved animals (Britton et al. 2002). Additionally, PI3K activity is modulated in response to starvation (Fig. 7A+B). In larvae, hyperactivation of the dInR pathway leads to increased accumulation of nutrients, while inhibiting PI3K activity depletes stored nutrients from the fat body (Britton et al. 2002). Thus, like in mammals, insulin acts as a strong anabolic inducer in the *Drosophila* fat body, an organ that resembles the mammalian liver as the principal site of stored glycogen (Wigglesworth 1949). Even under adverse nutritional conditions *dInR*/*PI3K* expressing cells continue stockpiling nutrients and grow, which induces hypersensitivity to starvation of the whole larva (Britton et al. 2002). Like *dInR* overexpressing larvae, *Imp-L2* mutants display enhanced dInR signaling activity and show a massive increase in lethality when nutrients become limiting (Fig. 6D). Hence, we propose that Imp-L2 is necessary to block insulin signaling under starvation conditions by regulating Dilp activity. Whereas the expression of *dilp3* and *5* is directly suppressed at the transcriptional level in the m-NSCs by starvation (Ikeya et al. 2002), it is not known how Dilp2, the most potent inducer of growth, is regulated under starvation. Imp-L2 provides a new way of controlling Dilp2 activity under adverse nutritional conditions, as it is induced and necessary under starvation, and by binding to Dilp2 counteracts its function.

The fact that the lethality of *Imp-L2* mutant larvae is not much increased when raised on an amino acid- (aa) free sugar source (20% glucose in Fig. 6D) suggests that the insulin inhibiting effect of Imp-L2 is mainly subject to sugar availability. When the glucose concentration is lowered to 1%, *Imp-L2* mutant larvae die to the same extent as if raised on PBS only. Thus, lacking sugar triggers a response that downregulates insulin signaling in an Imp-L2-dependent manner.

It has been shown that the CC cells, unlike the m-NSCs or any other group of cells known to regulate hemolymph glucose, express *Drosophila* cognates of sulphonylurea receptor (Sur)

and potassium channel (Ir) proteins (Kim and Rulifson 2004). Sur and Ir comprise ATP-sensitive potassium channels regulating hormone secretion by islets and other mammalian glucose sensing cells (Aguilar-Bryan et al. 1995; Miki et al. 2001; Seino and Miki 2003). Blocking the K_{ATP} channels of the CC by the drugs glyburide and tolbutamide results in enhanced hormonal secretion and an up to 40% increase in hemolymph glucose levels (Kim and Rulifson 2004). The increase in hemolymph glucose has so far believed to be solely due to enhanced secretion of the adipokinetic hormone (Akh), a polypeptide with glucagon-like functions that is produced and secreted exclusively by CC cells in *Drosophila* (Van der Horst 2003; Kim and Rulifson 2004; Lee and Park 2004). Since ectopic expression of Akh only partly rescues the hypoglycemic effects of CC ablation (Kim and Rulifson 2004), we propose that the lack of CC-produced Imp-L2 accounts for the rest of the hypoglycemia. Through its negative regulation of Dilp2 activity, which itself is sufficient to rescue the hyperglycemia induced by m-NSC ablation (Rulifson et al. 2002), Imp-L2 possesses the ability to exert an additional effect on the control of hemolymph glucose levels. Further studies about the changes of hemolymph glucose levels in genetic backgrounds with varying Dilp2 and Imp-L2 levels should clarify the role of Imp-L2 in glucose metabolism.

Materials and Methods

Fly stocks

The following fly stocks and transgenes have been used for this study: *y w* ; w^{1118} ; GMR-*Gal4* (gift of M. Freeman, MRC Laboratory of Molecular biology, Cambridge, UK); C10-*Gal4* (corresponds to C765-*Gal4*, (Nellen et al. 1996)), arm-*Gal4*, Act5C-*Gal4*, , UAS-*GFP*, UAS-*lacZ* (all from the Bloomington *Drosophila* stock center); ppl-*Gal4* (gift from M. Pankratz, Institut für Genetik, Karlsruhe, Germany); UAS-*dInR* (Brogiolo et al. 2001); Df(3L)AC1 (Brogiolo et al. 2001); tGPH (Britton et al. 2002); GMR>w^{+}>*Gal4* (Brogiolo et al. 2001); UAS-*dPTEN* (Huang et al. 1999); UAS-*dilp2* (Brogiolo et al. 2001); GE24013 (GenExel Inc.).
All crosses were performed at 25°C if not stated otherwise.

EP-screen and isolation of Imp-L2 alleles

The EP screen that led to the identification of *Imp-L2* will be described elsewhere (F. Wittwer, W. Brogiolo, H. Stocker, D. Nellen, K. Basler, E. Hafen, unpublished data). An EP element was identified in the *Imp-L2* locus suppressing the GMR-*Gal4*, UAS-*InR* induced big eye phenotype. Plasmid rescue of EP5.66 revealed that it was inserted 6'969bps upstream of the first exon of the *Imp-L2-RB* (CG15009-RB) transcript.
To obtain loss-of-function alleles of *Imp-L2*, we performed an EMS mutagenesis screen in which we selected mutated chromosomes carrying EP5.66 that were no longer able to suppress the *dInR* overexpression phenotype in the eye. Therefore, EP5.66 males were fed with 25mM EMS (ethylmethansulfonate) and subsequently crossed to GMR-*Gal4*, UAS-*dInR* virgins. 39'000 F1 flies were screened for a reversion of the suppressive effect of EP5.66 on the growth phenotype caused by GMR-*Gal4*, UAS-*dInR*. Only one of the identified revertant lines, $Imp\text{-}L2^{MG2}$, could be confirmed. By sequencing the genomic DNA of $Imp\text{-}L2^{MG2}$, a point mutation that resulted in an amino acid exchange (Trp232Stop) was found.
In order to generate further *Imp-L2* mutants, we mobilized the P-element GE24013 (w^+ marked) inserted 102bp's upstream of the first exon of the Imp-L2-RC transcript by supplying Δ2-3 transposase. Jump starter males were mated with balancer females, and single F1 w^- males were recrossed to balancer virgins. 350 stocks were established and molecularly tested for deletions by single-fly PCR using several primer pairs revealing the alleles $Imp\text{-}L2^{Def42}$, $Imp\text{-}L2^{Def20}$, $Imp\text{-}L2^{Def35}$, $Imp\text{-}L2^{Def223}$, and $Imp\text{-}L2^{Def29}$. Because GE24013 contained various polymorphisms in *Imp-L2*, of which several resulted in amino acid substitutions compared to the published *Imp-L2* sequence, a precise jump-out line of GE24013 could not be used as a control in the loss-of-function experiments.

Construction of plasmids

In order to generate the UAS-*l.IMPL2* construct, a BglII/XhoI fragment of *Imp-L2* was cut out of the *Imp-L2-RB* containing cDNA clone LP06542 and inserted into the pUAST vector (Brand and Perrimon 1993). To obtain UAS-*s.IMPL2*, we PCR amplified the second and third exon of *Imp-L2* from genomic DNA, a region shared by all three *Imp-L2* transcripts (primer sequences available on request). The fragment was subcloned into the pCRII-Topo vector (Invitrogen). The insert was then cut out by EcoRI and cloned into the pUAST vector (Brand

and Perrimon 1993). UAS-*IMPL2-RA* was constructed by inserting a SalI/EcoRI fragment of the cDNA clone SD10052 into the pUAST vector. To generate UAS-*IMPL2-RC*, an EcoRI/XhoI fragment from the cDNA clone SD23735 was inserted into the pUAST vector. For the generation of the genomic rescue construct, we used a genomic fragment, L2G314 kindly provided by J. Natzle (Department of Molecular and Cellular Biology, University of California, Davis, US). The fragment contains 5kb of genomic sequence upstream of the first exon of the Imp-L2-RB transcript, and 1kb downstream of the third exon that was excised by BamHI/Asp718 and inserted into a pCaSpeR-4 vector (Thummel 1992).
The *Flag-dilp2* construct was created by PCR amplifying the *dilp2* coding region without the signal peptide sequence from the full-length cDNA clone, EST GH11579 (obtained from Research Genetics), introducing a single flag tag at the 5' end (primer sequences available on request). Further, the primers contained EcoRI and XhoI restriction sites. The resulting PCR product was then cloned as an EcoRI/XhoI fragment into an PS261 vector containing the hemagglutinin signal peptide sequence. From PS261, the *Flag-dilp2* with the signal peptide was PCR amplified to introduce BglII and XbaI restriction sites. The BglII/XbaI fragment was inserted into a pUAST vector (Brand and Perrimon 1993).

Cell culture

Drosophila embryonic S2 cells were grown at 25°C in Schneider's Drosophila medium (Gibco/Invitrogen) supplemented with 10% heat-inactivated fetal-calf serum (FCS), as well as Penicillin and Streptomycin.
For the construction of the stably expressing *Flag-dilp2* cell line, S2 cells were co-transfected with UAS-*Flag-dilp2*, Act-*Gal4*, and a third vector containing a Blasticidin resistance gene, using effectene transfection reagent (Qiagen). Two days after the transfection, the selection medium (Schneider's containing 10% FCS and 1:200 5mg/ml Blasticidin) was added to the cells. After ten days the selection medium was replaced by Schneider's containing 10% FCS and 1:500 5mg/ml Blasticidin.

In vitro pulldown assay

S2 cells expressing *Flag-dilp2* were grown to confluency on 175 cm2 culture flasks, washed with ice-cold PBS and extracted in IP buffer (120mM NaCl, 50mM Tris, pH 7.5, 20mM NaF, 1mM benzamidine, 1 mM EDTA, 6mM EGTA, 15mM $Na_4P_2O_7$, 0.5% Nonidet P-40, 30mM β-Glycerolphosphate, 1x Complete Mini Protease Inihibitor (Roche)). After incubation for 15 min on an orbital shaker at 4°C, solubilized material was recovered by centrifugation at 13.000 rpm for 15 min and supernatants were collected. 5µg of anti-flag-Antibody (Sigma M2, F3165) was added and incubated over night at 4°C while rotating. Protein G sepharose beads (Amersham Biosciences) were added for 2 h and the beads washed 4x with IP buffer. To verify the immunoprecipitation, a fraction of the beads was incubated with SDS loading buffer (62,5mM Tris-HCl, pH 6,8, 20mM DTT, 2% SDS, 25% glycerol, 0,02% bromophenol blue) for 5 min at 90°C and the proteins were separated by SDS PAGE. The presence of Flag-Dilp2 was confirmed by immunoblotting.
For the *in vitro* translation the *Imp-L2-RC* cDNA (SD23735) was cloned into the pCRII.1 vector (Invitrogen) downstream of the SP6 polymerase promoter. Imp-L2 was translated *in vitro* using the TNT Quick coupled Transcription/Translation System (Promega) according to the manufacturer's protocol. Briefly, 2µg of DNA was incubated with 20µCi [^{35}S]methionine and 20µl TNT Quick Master Mix in a total volume of 25µl for 90 min at 30°C. 2.5µl of the product was used in the *in vitro* pulldown assay together with Flag-Dilp2 bound to beads or Protein G beads alone in IP buffer containing 0.05% NP-40. The reaction was rotated over night at 4°C, the beads were washed 6x with IP buffer (0.05% NP-40) and incubated with SDS loading buffer containing 100mM DTT for 10 min at 80°C. The dissociated proteins were separated using SDS-PAGE and detected by autoradiography.

Phenotypic analyses

Freshly eclosed flies were collected, separated according to sex, placed on normal fly food for 72h, and anesthetized for 1 minute with ether prior to weighing. Weight was determined using a Mettler Toledo MX5 microbalance.
Wing size was analyzed as described earlier (Reiling and Hafen 2004). ImageJ 1.32j software was used to determine the pixels of the wing area.

Scanning electron microscope pictures were taken from adult flies that were critical-point dried and coated with gold.
Heat-shock induced overexpression clones (*y*, *w*, hs-*Flp*; GMR>w^+>*Gal4*) were induced 24-48h after egg laying by a 1h heat-shock at 37°C. Adult fly heads were cut in half using a razor blade and briefly stored on ice. Eyes were then fixed as described (Basler et al. 1991).
For the analysis of the ovaries, freshly eclosed, non-virgin females were collected, and placed for 72h on normal fly food. The ovaries of ten females of each genotype were dissected in PBS, whereby the ovarioles, mature eggs, and stage 10 egg chambers were counted for each ovary separately.

Starvation experiments

For all starvation experiments, eggs were collected for 2h on apple agar plates supplemented with yeast. After 72h larvae were quickly washed in PBS, and transferred either to a new apple agar plate with yeast ("normal food" or "yeast"), a solution containing 20% glucose in PBS, or a filter paper soaked with 1% glucose in PBS or PBS only. After 24h, dead larvae were counted or dissected for immunohistochemistry.
For the tGPH reporter analysis under starvation, the "PBS" or "yeast" conditions were used (see above). After 4h starvation larvae were dissected in PBS, fixed, and stained with Hoechst. Pictures were taken using a Leica SP2 confocal laser scanning microscope.

Immunohistochemistry and *in situ* hybridization

The antibody (Ab) against Imp-L2 was described earlier (Garbe et al. 1993), and kindly provided by J. Natzle (Department of Molecular and Cellular Biology, University of California, Davis, US). Antibody staining against Imp-L2 was performed using the following dilutions: rat-anti-Imp-L2 (1:200), donkey-anti-rat-FITC (1:400, Jackson). Other Abs used were: anti-β-Galactosidase (1:,), an Ab against the C terminus of dInR (INRcT, 1:10'000)(Fernandez et al. 1995). Nuclei were either stained with DAPI or Hoechst. Pictures were taken using a Leica SP2 confocal laser scanning microscope.

RNA *in situ* hybridization using DIG-labeled probes was performed as described (Brogiolo et al. 2001). The probes against *Imp-L2* were derived from the *s.Imp-L2* in a pBluescript SK^+ vector.

References

Aguilar-Bryan, L., Nichols, C.G., Wechsler, S.W., Clement, J.P.t., Boyd, A.E., 3rd, Gonzalez, G., Herrera-Sosa, H., Nguy, K., Bryan, J., and Nelson, D.A. 1995. Cloning of the beta cell high-affinity sulfonylurea receptor: a regulator of insulin secretion. *Science* **268**(5209): 423-426.

Basler, K., Christen, B., and Hafen, E. 1991. Ligand-independent activation of the sevenless receptor tyrosine kinase changes the fate of cells in the developing Drosophila eye. *Cell* **64**(6): 1069-1081.

Bohni, R., Riesgo-Escovar, J., Oldham, S., Brogiolo, W., Stocker, H., Andruss, B.F., Beckingham, K., and Hafen, E. 1999. Autonomous control of cell and organ size by CHICO, a Drosophila homolog of vertebrate IRS1-4. *Cell* **97**(7): 865-875.

Boisclair, Y.R., Rhoads, R.P., Ueki, I., Wang, J., and Ooi, G.T. 2001. The acid-labile subunit (ALS) of the 150 kDa IGF-binding protein complex: an important but forgotten component of the circulating IGF system. *J Endocrinol* **170**(1): 63-70.

Brand, A.H. and Perrimon, N. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**(2): 401-415.

Britton, J.S., Lockwood, W.K., Li, L., Cohen, S.M., and Edgar, B.A. 2002. Drosophila's insulin/PI3-kinase pathway coordinates cellular metabolism with nutritional conditions. *Developmental Cell* **2**(2): 239-249.

Brogiolo, W., Stocker, H., Ikeya, T., Rintelen, F., Fernandez, R., and Hafen, E. 2001. An evolutionarily conserved function of the Drosophila insulin receptor and insulin-like peptides in growth control. *Curr Biol* **11**(4): 213-221.

Burger, A.M., Zhang, X., Li, H., Ostrowski, J.L., Beatty, B., Venanzoni, M., Papas, T., and Seth, A. 1998. Down-regulation of T1A12/mac25, a novel insulin-like growth factor binding protein related gene, is associated with disease progression in breast carcinomas. *Oncogene* **16**(19): 2459-2467.

Butler, A.A. and Le Roith, D. 2001. Control of growth by the somatropic axis: growth hormone and the insulin-like growth factors have related and independent roles. *Annual Review of Physiology* **63**: 141-164.

Cao, C. and Brown, M.R. 2001. Localization of an insulin-like peptide in brains of two flies. *Cell & Tissue Research* **304**(2): 317-321.

Casey, O.M., Fitzpatrick, R., McInerney, J.O., Morris, D.G., Powell, R., and Sreenan, J.M. 2004. Analysis of gene expression in the bovine corpus luteum through generation and characterisation of 960 ESTs. *Biochim Biophys Acta* **1679**(1): 10-17.

Chen, C., Jack, J., and Garofalo, R.S. 1996. The Drosophila insulin receptor is required for normal growth. *Endocrinology* **137**(3): 846-856.

Colombani, J., Bianchini, L., Layalle, S., Pondeville, E., Dauphin-Villemant, C., Antoniewski, C., Carre, C., Noselli, S., and Leopold, P. 2005. Antagonistic actions of ecdysone and insulins determine final size in Drosophila. *Science* **310**(5748): 667-670.

Colombani, J., Raisin, S., Pantalacci, S., Radimerski, T., Montagne, J., and Leopold, P. 2003. A nutrient sensor mechanism controls Drosophila growth. *Cell* **114**(6): 739-749.

Dominguez, F., Avila, S., Cervero, A., Martin, J., Pellicer, A., Castrillo, J.L., and Simon, C. 2003. A combined approach for gene discovery identifies insulin-like growth factor-binding protein-related protein 1 as a new gene implicated in human endometrial receptivity. *J Clin Endocrinol Metab* **88**(4): 1849-1857.

Drummond-Barbosa, D. and Spradling, A.C. 2001. Stem cells and their progeny respond to nutritional changes during Drosophila oogenesis. *Dev Biol* **231**(1): 265-278.

Fernandez, R., Tabarini, D., Azpiazu, N., Frasch, M., and Schlessinger, J. 1995. The Drosophila insulin receptor homolog: a gene essential for embryonic development encodes two receptor isoforms with different signaling potential. *Embo J* **14**(14): 3373-3384.

Garbe, J.C., Yang, E., and Fristrom, J.W. 1993. IMP-L2: an essential secreted immunoglobulin family member implicated in neural and ectodermal development in Drosophila. *Development* **119**(4): 1237-1250.

Garofalo, R.S. 2002. Genetic analysis of insulin signaling in Drosophila. *Trends Endocrinol Metab* **13**(4): 156-162.

Huang, H., Potter, C.J., Tao, W., Li, D.M., Brogiolo, W., Hafen, E., Sun, H., and Xu, T. 1999. PTEN affects cell size, cell proliferation and apoptosis during Drosophila eye development. *Development* **126**(23): 5365-5372.

Hwa, V., Oh, Y., and Rosenfeld, R.G. 1999. The insulin-like growth factor-binding protein (IGFBP) superfamily. *Endocrine Reviews* **20**(6): 761-787.

Ikeya, T., Galic, M., Belawat, P., Nairz, K., and Hafen, E. 2002. Nutrient-dependent expression of insulin-like peptides from neuroendocrine cells in the CNS contributes to growth regulation in Drosophila. *Curr Biol* **12**(15): 1293-1300.

Jones, J.I. and Clemmons, D.R. 1995. Insulin-like growth factors and their binding proteins: biological actions. *Endocr Rev* **16**(1): 3-34.

Kim, S.K. and Rulifson, E.J. 2004. Conserved mechanisms of glucose sensing and regulation by Drosophila corpora cardiaca cells. *Nature* **431**(7006): 316-320.

Lee, G. and Park, J.H. 2004. Hemolymph sugar homeostasis and starvation-induced hyperactivity affected by genetic manipulations of the adipokinetic hormone-encoding gene in Drosophila melanogaster. *Genetics* **167**(1): 311-323.

Melcher, C. and Pankratz, M.J. 2005. Candidate gustatory interneurons modulating feeding behavior in the Drosophila brain. *PLoS Biol* **3**(9): e305.

Miki, T., Liss, B., Minami, K., Shiuchi, T., Saraya, A., Kashima, Y., Horiuchi, M., Ashcroft, F., Minokoshi, Y., Roeper, J., and Seino, S. 2001. ATP-sensitive K+ channels in the hypothalamus are essential for the maintenance of glucose homeostasis. *Nat Neurosci* **4**(5): 507-512.

Montagne, J., Stewart, M.J., Stocker, H., Hafen, E., Kozma, S.C., and Thomas, G. 1999. Drosophila S6 kinase: a regulator of cell size. *Science* **285**(5436): 2126-2129.

Natzle, J.E., Hammonds, A.S., and Fristrom, J.W. 1986. Isolation of genes active during hormone-induced morphogenesis in Drosophila imaginal discs. *Journal of Biological Chemistry* **261**(12): 5575-5583.

Nellen, D., Burke, R., Struhl, G., and Basler, K. 1996. Direct and long-range action of a DPP morphogen gradient. *Cell* **85**(3): 357-368.

Osterbur, D.L., Fristrom, D.K., Natzle, J.E., Tojo, S.J., and Fristrom, J.W. 1988. Genes expressed during imaginal discs morphogenesis: IMP-L2, a gene expressed during imaginal disc and imaginal histoblast morphogenesis. *Developmental Biology* **129**(2): 439-448.

Rajah, R., Khare, A., Lee, P.D., and Cohen, P. 1999. Insulin-like growth factor-binding protein-3 is partially responsible for high-serum-induced apoptosis in PC-3 prostate cancer cells. *J Endocrinol* **163**(3): 487-494.

Reiling, J.H. and Hafen, E. 2004. The hypoxia-induced paralogs Scylla and Charybdis inhibit growth by down-regulating S6K activity upstream of TSC in Drosophila. *Genes Dev* **18**(23): 2879-2892.

Rulifson, E.J., Kim, S.K., and Nusse, R. 2002. Ablation of insulin-producing neurons in flies: growth and diabetic phenotypes. *Science* **296**(5570): 1118-1120.

Saltiel, A.R. and Kahn, C.R. 2001. Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* **414**(6865): 799-806.

Sarbassov, D.D., Guertin, D.A., Ali, S.M., and Sabatini, D.M. 2005. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* **307**(5712): 1098-1101.

Seino, S. and Miki, T. 2003. Physiological and pathophysiological roles of ATP-sensitive K+ channels. *Prog Biophys Mol Biol* **81**(2): 133-176.

Sloth Andersen, A., Hertz Hansen, P., Schaffer, L., and Kristensen, C. 2000. A new secreted insect protein belonging to the immunoglobulin superfamily binds insulin and related peptides and inhibits their activities. *J Biol Chem* **275**(22): 16948-16953.

Stewart, C.E. and Rotwein, P. 1996. Growth, differentiation, and survival: multiple physiological functions for insulin-like growth factors. *Physiol Rev* **76**(4): 1005-1026.

Takahashi, Y., Kadowaki, H., Momomura, K., Fukushima, Y., Orban, T., Okai, T., Taketani, Y., Akanuma, Y., Yazaki, Y., and Kadowaki, T. 1997. A homozygous kinase-defective mutation in the insulin receptor gene in a patient with leprechaunism. *Diabetologia* **40**(4): 412-420.

Taylor, S.I. 1992. Lilly Lecture: molecular mechanisms of insulin resistance. Lessons from patients with mutations in the insulin-receptor gene. *Diabetes* **41**(11): 1473-1490.

Thummel, C.S., and Pirrotta, V. 1992. New pCaSpeR P element vectors. *Dros Inf Serv* **71**: 150.

Van der Horst, D.J. 2003. Insect adipokinetic hormones: release and integration of flight energy metabolism. *Comparative Biochemistry & Physiology Part B, Biochemistry & Molecular Biology* **136**(2): 217-226.

Wandji, S.A., Gadsby, J.E., Barber, J.A., and Hammond, J.M. 2000. Messenger ribonucleic acids for MAC25 and connective tissue growth factor (CTGF) are inversely regulated during folliculogenesis and early luteogenesis. *Endocrinology* **141**(7): 2648-2657.

Wigglesworth, V.B. 1949. The utilization of reserve substances in Drosophila during flight. *J Exp Biol* **26**(2): 150-163, illust.

Wittwer, F., Jaquenoud, M., Brogiolo, W., Zarske, M., Wustemann, P., Fernandez, R., Stocker, H., Wymann, M.P., and Hafen, E. 2005. Susi, a negative regulator of Drosophila PI3-kinase. *Dev Cell* **8**(6): 817-827.

Yamanaka, Y., Wilson, E.M., Rosenfeld, R.G., and Oh, Y. 1997. Inhibition of insulin receptor activation by insulin-like growth factor binding proteins. *Journal of Biological Chemistry* **272**(49): 30729-30734.

Zapf, J. 1995. Physiological role of the insulin-like growth factor binding proteins. *Eur J Endocrinol* **132**(6): 645-654.

Zinke, I., Schutz, C.S., Katzenberger, J.D., Bauer, M., and Pankratz, M.J. 2002. Nutrient control of gene expression in Drosophila: microarray analysis of starvation and sugar-dependent response. *Embo J* **21**(22): 6162-6173.

Figures

Figure 1. *Imp-L2* overexpression suppresses *dInR* induced growth

(A-E) Shown are SEMs of female fly eyes (magnification=300x). Overexpression of *Imp-L2* by EP5.66 (B) suppresses the *dInr* dependent big eye phenotype (A). Flies carrying a point mutation in the *Imp-L2* gene that leads to a premature stop (*Imp-L2*^{MG2}) , identified in an EMS reversion mutagenesis, no longer suppressed the *dInR* induced phenotype (C). UAS transgenes containing either the full-length *Imp-L2-RB* transcript (UAS-*l.IMPL2*, D) or one lacking the first exon (UAS-*s.IMPL2*, E), are also able to rescue the overgrowth caused by overexpression of *dInR*. Genotypes: *y*, *w*; GMR-*Gal4*, UAS-*dInR*/UAS-*GFP* (A), *y*, *w*; GMR-*Gal4*, UAS-*dInR*/+ ; EP5.66/+ (B), *y*, *w*; GMR-*Gal4*, UAS-*dInR*/+ ; EP5.66, *Imp-L2*^{MG2}/+ (C), *y*, *w*; GMR-*Gal4*, UAS-*dInR*/+ ; UAS-*l.IMPL2*/+ (D), *y*, *w*; GMR-*Gal4*, UAS-*dInR*/+ ; UAS-*s.IMPL2*/+ (E). (F) Genomic organization of the Imp-L2 locus. Indicated are the mutant alleles and used P-elements. MG2 marks the point mutation in the EMS allele *Imp-L2*^{MG2}. (G) Alignment of Imp-L2, its orthologs in lower organisms and the putative human ortholog. Dark and grey boxes indicate amino acid identity and similarity, respectively. The triangle marks the tryptophan mutated to a stop in *Imp-L2*^{MG2}. Asterisks mark the cysteins forming the two disulfide bridges. Dm: *Drosophila melanogaster* Imp-L2, Ag: *Anopheles gambiae* CP2953, Sf: *Spodoptera frugiperda* IBP, Ce: *Caenorhabditis elegans* zig-4, Hs: *Homo sapiens* IGFBP-rP1.

Figure 2. Tissue specific overexpression of *Imp-L2* reduces growth non-autonomously

(A) Tangential section through an eye containing an *Imp-L2* overexpression clone, marked by the lack of red pigment. While differentiation and patterning of the *Imp-L2* overexpressing cells seems completely normal, cell size is reduced, with the cells being bigger the farther away from the clone they are. (B) Eye-specific overexpression of UAS-*s.IMPL2* reduces total body weight (-38.3%). The graph shows male body weight. (C) Overexpression of UAS-*l.IMPL2* by the fat body specific ppl-*Gal4* driver results in a 56.1% weight reduction in male flies (D). (E) Overexpression of different *Imp-L2* UAS transgenes in the wing reduces total wing area due to a decrease in cell number and, except for UAS-*l.IMPL2*, also cell size.

Shown are female wings. All experiments were repeated at least twice (*p<0.0001 when compared to the "GFP" controls).
Genotypes: (A) *y*, *w*, hs-*flp/y, w*; GMR>*w*+>*Gal4*/+; UAS-*l.IMPL2*/+, (B) "white" bar *y*, *w*; GMR-*Gal4*/+; UAS-*s.IMPL2*/+, "black" bar *y*, *w*; GMR-*Gal4*/UAS-*GFP*, (C+D) left fly and "white" bar: *y*, *w*; ppl-*Gal4*/+; UAS-*l.IMPL2*/+, right fly and "black" bar: *y*, *w*; ppl-*Gal4*/UAS-*GFP*, (E) "GFP": *y*, *w*; UAS-*GFP*/+; C10-*Gal4*/+, "l.IMPL2": *y*, *w*;; C10-*Gal4*/UAS-*l.IMPL2*, "l.IMPL2-3T": *y*, *w*; UAS-*l.IMPL2-3T(34)*/+; C10-*Gal4*/+, "IMPL2-RA": *y*, *w*; UAS-*IMPL2-RA*(10)/+; C10-*Gal4*/+, "IMPL2-RC": *y*, *w*;; C10-*Gal4*/UAS-*IMPL2-RC(4)*.

Figure 3. *Imp-L2* reduces PIP_3 levels

Shown are eye imaginal discs of 3rd instar larvae. Green displays the localization of the tGPH reporter (in A-D). Expression of dInR protein is shown in red (A'-D'), and nuclear DNA staining (DAPI) in blue (A'-D').
(A) In wild-type eye imaginal discs, *dInR*/*PI3K* signaling is low inhibiting tGPH reporter recruitment to the membrane. (B) Overexpression of dInR posterior to the morphogenetic furrow causes a strong membrane relocation of tGPH. (C) Simultaneous overexpression of *dInR* and *dPTEN* reduces membrane-bound tGPH partially. (D) Like *dPTEN*, *Imp-L2* overexpression reduces the *dInR* induced membrane localization. Although PIP_3 levels are strongly reduced when *dPTEN* or *Imp-L2* is co-overexpressed together with *dInR*, dInR protein levels are unaffected (B'-D').
Genotypes: (A, A') *y, w*; *tGPH*/+, (B, B') *y, w*; UAS-*dInR*, *tGPH*/+; GMR-*Gal4*/+, (C, C') *y, w*; UAS-*dInR*, *tGPH*/+; GMR-*Gal4*/UAS-*dPTEN*, (D, D') *y, w*; UAS-*dInR*, *tGPH*/+; GMR-*Gal4*/UAS-*s.IMPL2*.

Figure 4. *Imp-L2* is expressed in a specific pattern throughout development

(A) *In situ* hybridization with *Imp-L2* antisense probes in 3rd instar larvae strongly stains cells of the CC (filled arrowheads). (B) When stained longer, also several neurons of both brain hemispheres and the subesophagal ganglion show expression of Imp-L2 mRNA. (C) A cluster of cells with unknown function in the anterior midgut epithelium expresses *Imp-L2*. A higher

magnification shows that the Imp-L2 expressing cells are much smaller than the surrounding cells of the epithelium (C'). (D-I) Antibody (Ab) staining of larval and adult tissues with an Ab against Imp-L2 (green). (D+E) The CC (filled arrowheads) expresses Imp-L2 protein specifically at high levels. The corpora allata (CA) is innervated by Imp-L2 expressing axons (D). In the larval brain, specific neurons of both brain hemispheres and the sub-esophagal ganglion (white arrow) express Imp-L2 (D). Axon projections of the brain neurons expressing *Imp-L2* could be followed to central brain regions as well as the ring gland. Also larval midgut cells express Imp-L2 protein at high levels (F). In larvae carrying a copy of a dilp2-*lacZ* transgene, co-staining with an Imp-L2-Ab and an Ab against β-galactosidase revealed that the seven m-NSCs expressing four dilps also produce low levels of Imp-L2 (G). In the ovaries of adult females, in early stages of oogenesis, Imp-L2 is expressed in vesicle-like structures (H). Like in larvae, Imp-L2 is expressed in distinct neurons of both brain hemispheres of adult males and females (I).

Figure 5. Imp-L2 controls body and organ size

(A) Complete loss-of-*Imp-L2* function increases body size in males and females. (B) Total body weight is increased 27.5% in males and 63.9% in females. Introducing one copy of a genomic rescue construct reduces the size increase to the level of heterozygous *Imp-L2* mutants, which are also heavier (males 13.9% and females 43.6%) than control flies. *Imp-L2*$^{+/-}$ flies containing a copy of the genomic rescue construct are of nearly wild-type size. (C) The size increase of *Imp-L2*$^{-/-}$ flies is mainly due to an increase in cell number. (D) The additional size increase observed in *Imp-L2*$^{-/-}$ and *Imp-L2*$^{+/-}$ females is due to an increase in ovary size. Shown are the ovaries of non-virgin females. (E) While the amount of ovarioles is not changed in Imp-L2 mutants, the number of mature egg chambers per ovary is increased 135% in homozygous and 59% in heterozygous mutants when compared with controls. Apart from the mature eggs, *Imp-L2*$^{-/-}$ ovaries also display a 204% increased amount of stage 10 oocytes. (*$p<0.00001$).

Genotypes: "control" *y*, *w*/*w*, "Imp-L2$^{-/-}$" *y, w*;; *Imp-L2*^{Def42}/*Imp-L2*^{Def20}, "Imp-L2$^{+/-}$ in (B)" *y, w*;; *Imp-L2*^{Def20}/+, "Imp-L2$^{+/-}$in (C, D, E)" *y, w*;; *Imp-L2*^{Def42}/+, "Imp-L2$^{-/-}$, gen. rescue" *y, w*;; *Imp-L2*^{Def42}/*Imp-L2*^{Def20}, *Imp-L2*$^{genomic\ construct(57)}$, "Imp-L2$^{+/-}$, gen. rescue "*y, w*;; *Imp-L2*^{Def20}, *Imp-L2*$^{genomic\ construct(57)}$/+,

Figure 6. Imp-L2 binds Dilp2 and counteracts its activity

(A) The size increase of flies weakly overexpressing *dilp2* by arm-*Gal4* is enhanced dominantly by reducing Imp-L2 levels (*p<0.001). In a an *Imp-L2* loss-of-function background weak overexpression of *dilp2* results in lethality, which can be rescued by introducing a copy of the *Imp-L2* genomic rescue construct. (B) While overexpression of *dilp2* as well as *Imp-L2* at high levels by the fat body specific ppl-*Gal4* driver causes lethality, co-overexpression of *dilp2* and *Imp-L2* give rise to flies of wild-type size. (C) Imp-L2 binds Dilp2. Flag tagged Dilp2, extracted from stably transfected S2 cells, was bound to beads and incubated with *in-vitro*-translated, ^{35}S-labeled Imp-L2. Empty beads were used as a control. Imp-L2 could only be pulled down in the presence of Dilp2. (D) The survival rate of Imp-L2 mutant 3rd instar larvae is highly decreased under starvation. Larvae were subjected for 24 hours to different starvation conditions. While lethality of *Imp-L2*$^{-/-}$ larvae is only slightly increased when raised on 20% glucose, most of the larvae lacking *Imp-L2* die on 1% glucose and PBS.

Genotypes: (A) from left to right: (1) *y*, *w*; arm-*Gal4*/+; UAS-*dilp2*/+, (2) *y*, *w*; arm-*Gal4*/+; UAS-*dilp2*/*Imp-L2*^{Def42}, (3) *y*, *w*; arm-*Gal4*/+; UAS-*dilp2*, *Imp-L2*^{Def20}/*Imp-L2*^{Def42}, (4) *y*, *w*; arm-*Gal4*/UAS-*dilp2*; *Imp-L2*^{Def42}/*Imp-L2*^{Def20}, *Imp-L2*$^{genomic\ construct(57)}$, (5) *y, w*;; *Imp-L2*^{Def42}/*Imp-L2*^{Def20}, (6) *y, w*; arm-*Gal4*/UAS-*GFP*; *Imp-L2*^{Def42}/+, (7) *y*, *w*/*w*, (B) from left to right: (1) *y*, *w*; ppl-*Gal4*/+; UAS-*s.IMPL2*/UAS-*lacZ*, (2) *y*, *w*; ppl-*Gal4*/UAS-*dilp2*; UAS-*lacZ*/+, (3) *y*, *w*; ppl-*Gal4*/UAS-*dilp2*; UAS-*s.IMPL2*/+, (1) *y*, *w*; ppl-*Gal4*/+; UAS-*lacZ*/+, (D) "Imp-L2$^{-/-}$" *y, w*;; *Imp-L2*^{Def42}/*Imp-L2*^{Def20}, "Imp-L2$^{-/-}$, gen. rescue" *y, w*;; *Imp-L2*^{Def42}/*Imp-L2*^{Def20}, *Imp-L2*$^{genomic\ construct(57)}$, "Imp-L2$^{+/-}$" *y, w*;; *Imp-L2*^{Def42}/+, "control" *y*, *w*/*w*.

Figure 7. Imp-L2 is necessary for blocking dInR signaling under starvation

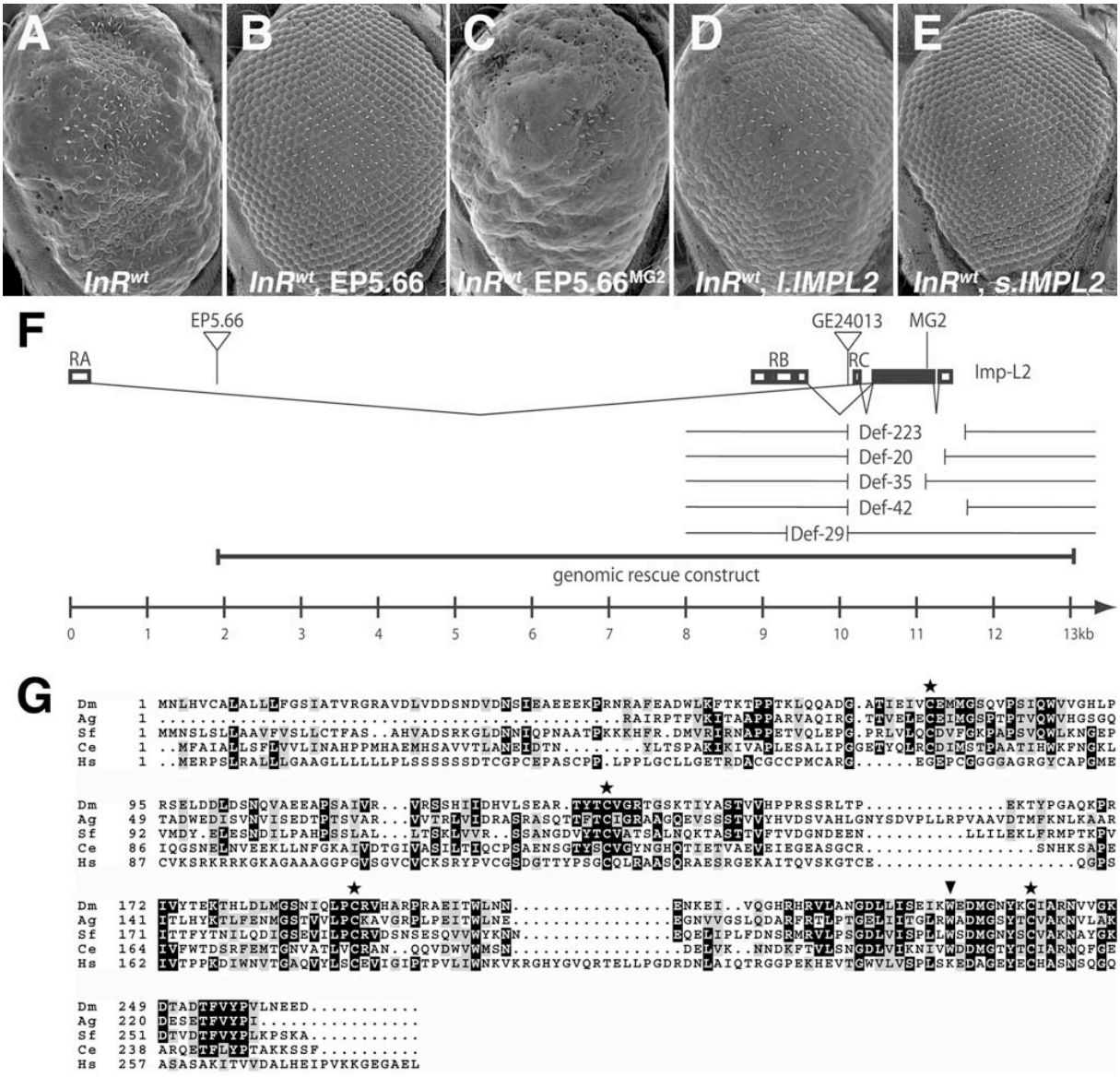
(A-D) tGPH fluorescence (green) in the fat body of feeding 3rd instar larvae under different nutrient conditions. (A'-D') Nuclear staining (Hoechst) is shown in blue.

While under normal conditions ("yeast") insulin signaling is already high in wild-type feeding 3rd instar larvae (A+A'), *Imp-L2* mutants display a further increase in PIP_3 localized to membranes of fat body cells (B+B''). After 4h on PBS, dInR signaling is reduced in wild-type controls (C+C''). In *Imp-L2*$^{-/-}$ larvae, although PIP_3 levels are slightly reduced upon

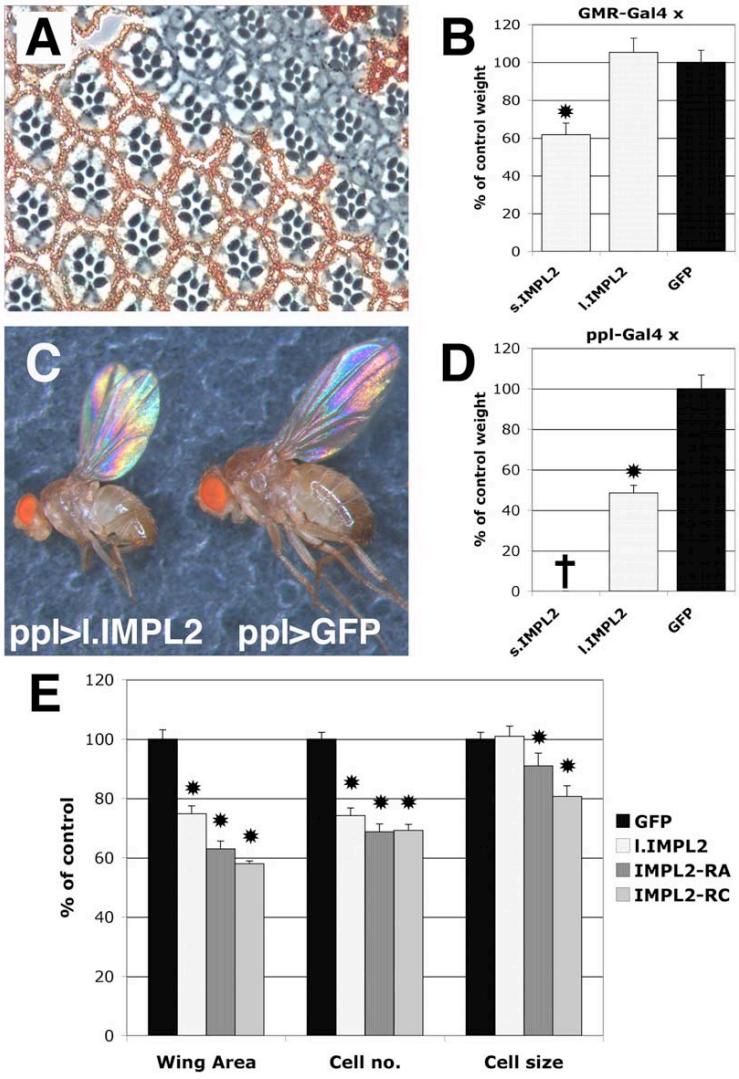
starvation (D+D'' compared with B+B''), PIP3 levels remain higher than in starved control larvae (compare D+D'' with A+A'').

(E+F) In starved control larvae (*yw*), Imp-L2 expression (green) is induced in fat body cells after 24h PBS-starvation. Imp-L2 is localized to vesicular-like structures not detectable under normal nutritional conditions.

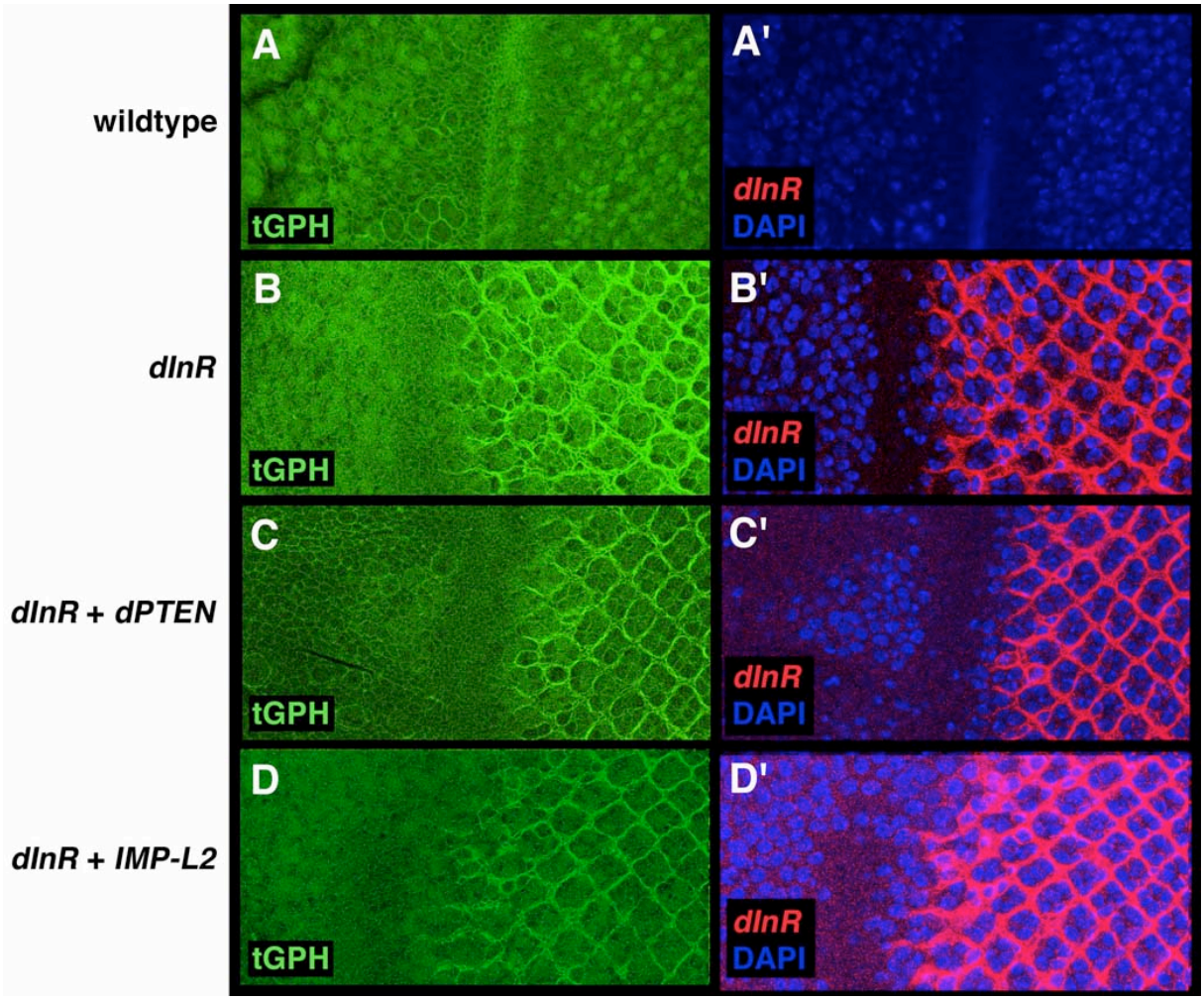
Genotypes: (A, C, E, and F) *y, w*, (B+D) *y, w*;; $Imp\text{-}L2^{Def42}/Imp\text{-}L2^{Def20}$.



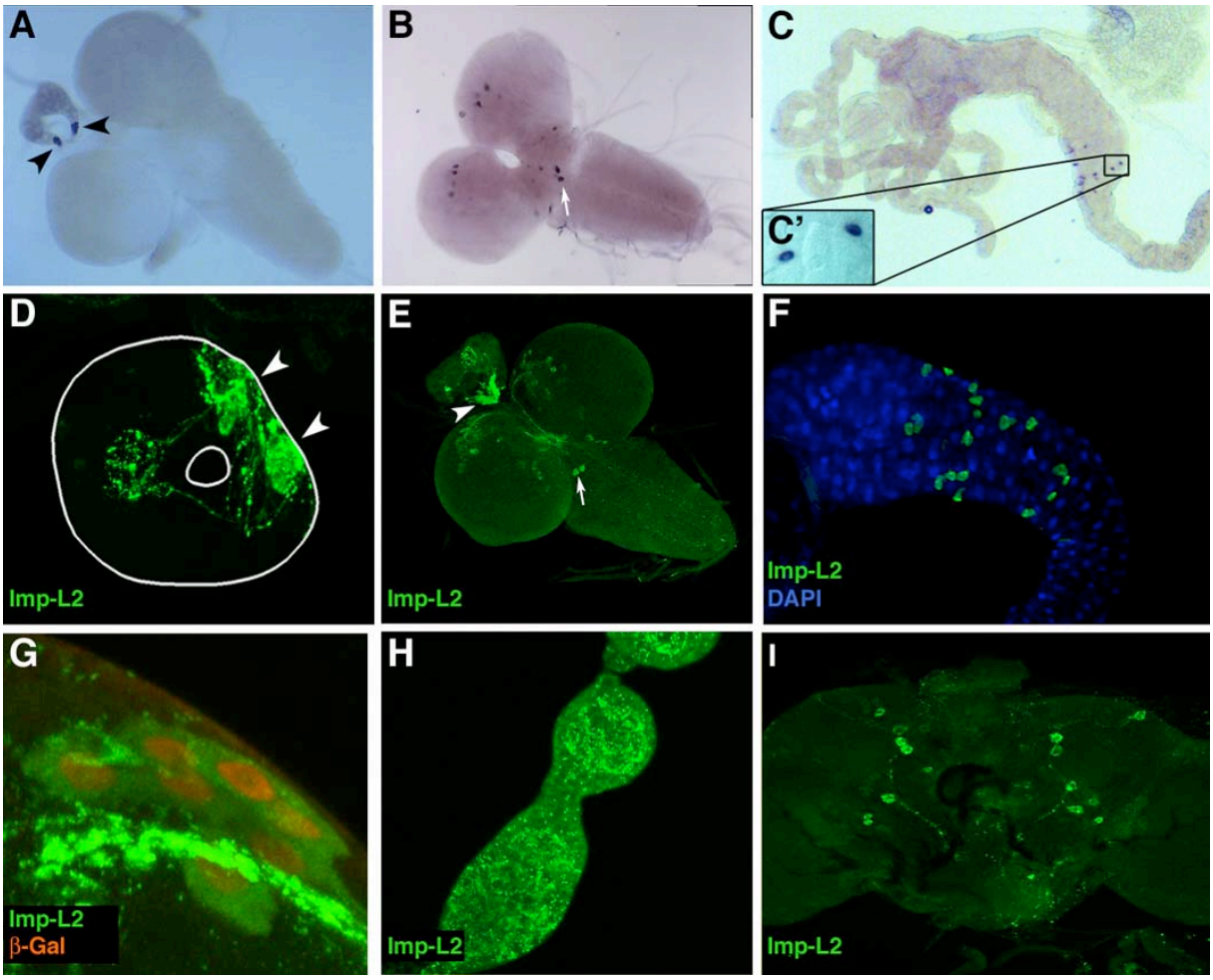
Honegger_Figure 1



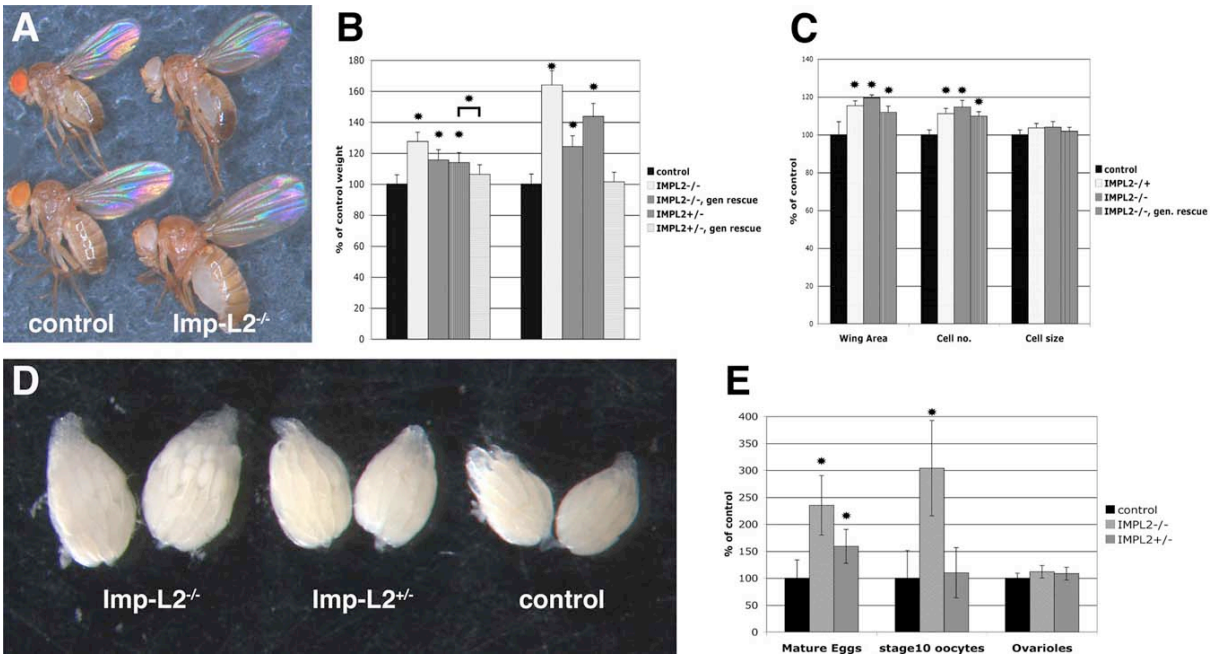
Honegger_Figure 2



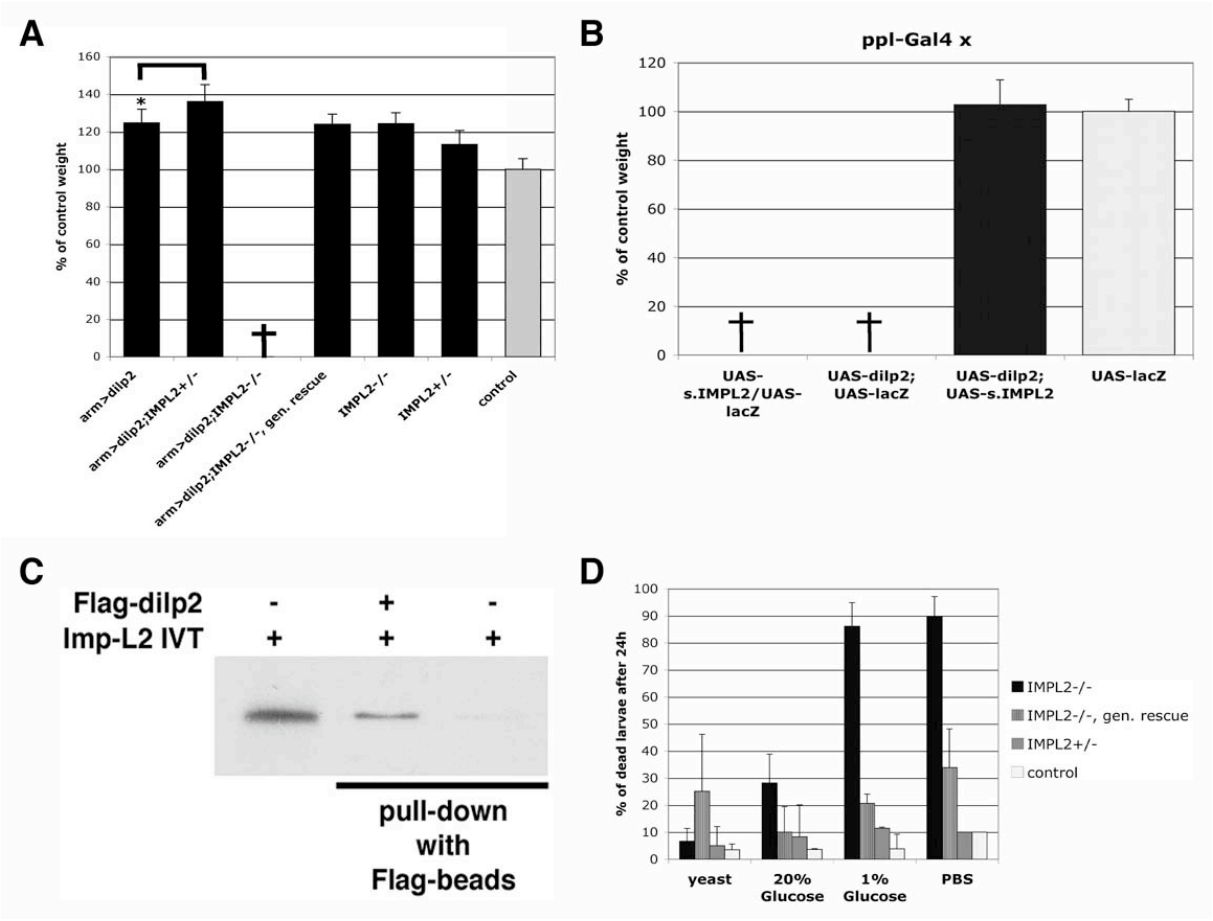
Honegger_Figure 3



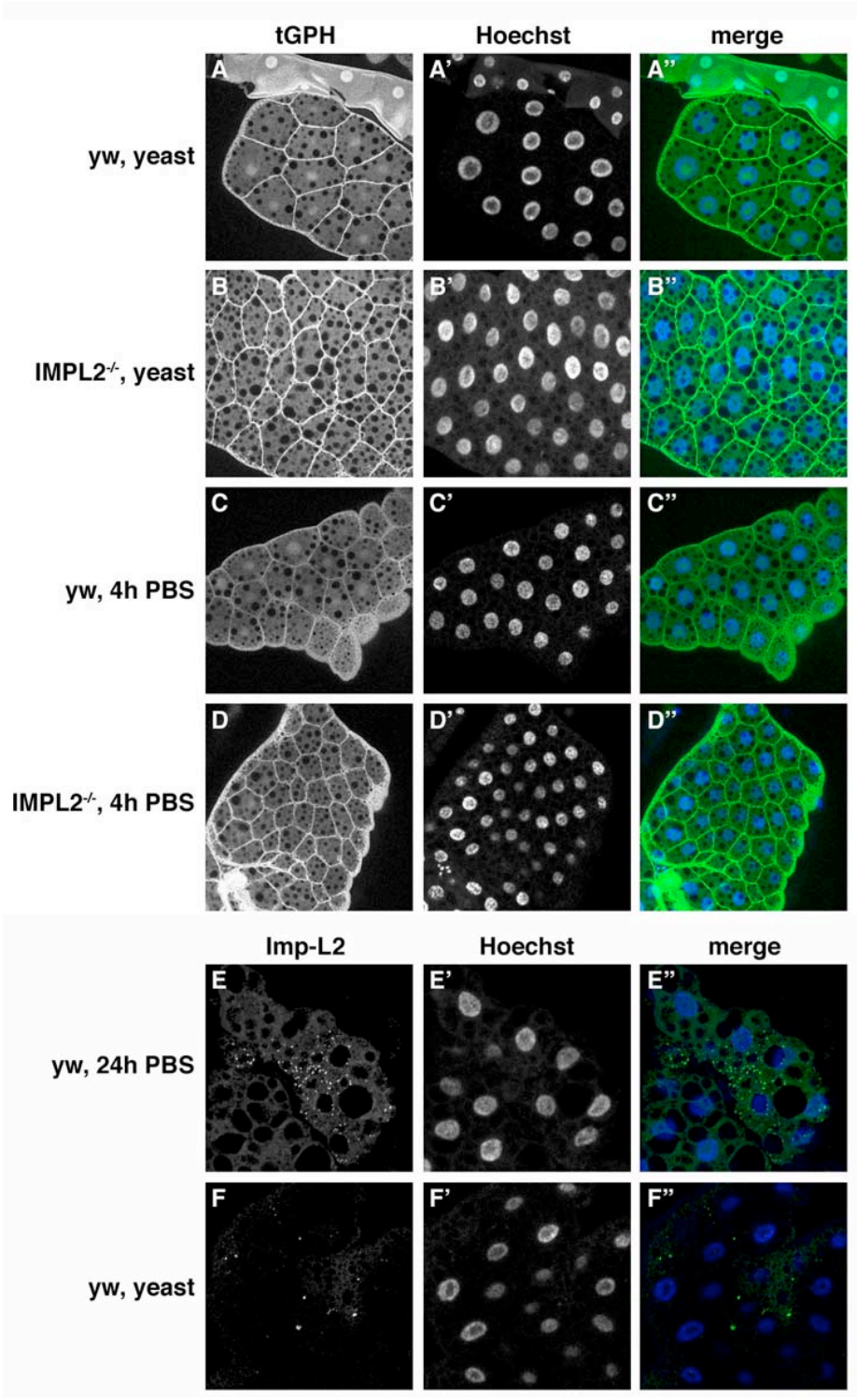
Honegger_Figure 4



Honegger_Figure 5



Honegger_Figure 6



Honegger_Figure 7

Supplemental results about Imp-L2

This chapter describes findings that were either mentioned as "data not shown" in the manuscript but will also cover experiments and results that have not been reported in (Honegger et al. 2006).

Imp-L2 gain-of-function analysis

Overexpression effects of different Imp-L2 constructs

As we have already shown in (Honegger et al. 2006), overexpression of UAS-*l.Imp-L2(1724)* by the fat body specific ppl-*Gal4* leads to a size reduction that is comparable to homozygous *chico* mutants. Ppl-*Gal4* driven overexpression of UAS-*s.IMPL2(1726)* causes lethality, though. To compare the overexpression phenotype of *l.Imp-L2* with the EP insertion in the *Imp-L2* locus, both EP5.66 and UAS-*l.IMPL2* were expressed under ppl-*Gal4* control (Fig. S1A). Surprisingly, even though the EP element contained the double amount of UAS sites, its effect on size, when driven by ppl-*Gal4*, was much weaker than with UAS-*l.IMPL2(1724)*. Ppl-*Gal4* was the only driver line with which EP5.66 led to weaker phenotypes than UAS-*l.IMPL2*. With all the other drivers tested, EP5.66 had stronger effects on size (Fig. S2A) (Honegger et al. 2006). Although the size decrease was weaker it could be fully attributed to the expression of *Imp-L2*, because driving the truncated version of *Imp-L2*, *Imp-L2*^{MG2} (which still contained the EP5.66), with ppl-*Gal4* had no longer any effect on total body weight (Fig. S1A). The reason for this discrepancy between EP5.66 and UAS-*l.IMPL2* when driven in the fat body has so far remained elusive.

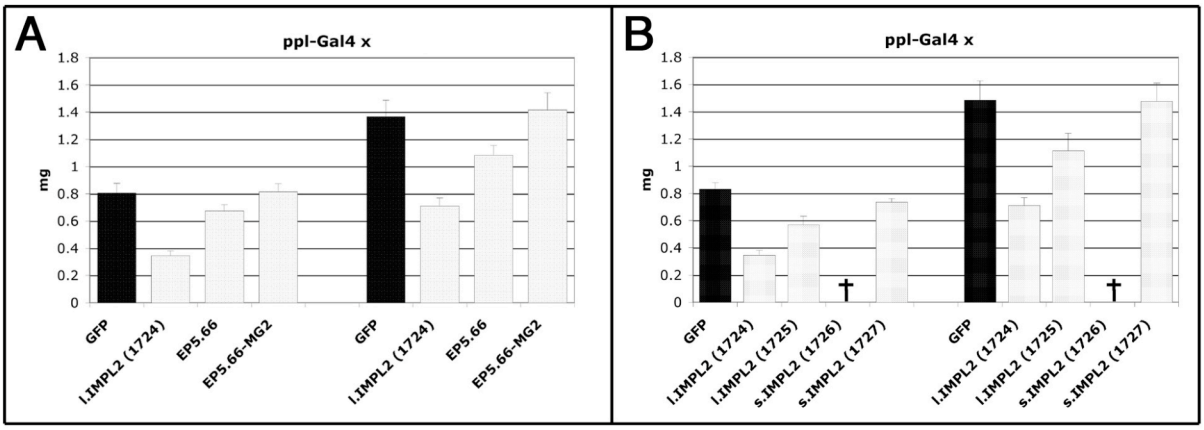


Figure S1: Analysis of different fat body specific *Imp-L2* overexpression.

Weights of 3 day-old adult flies, reared at 25°C. Males to the left, females to the right. (A) Imp-L2 expression in the fat body by ppl-*Gal4* using EP5.66, leads to a weaker size decrease than by UAS-*l.IMPL2*. Driving the truncated mutant version of *Imp-L2*, EP5.66, *Imp-L2*^{MG2}, by ppl-*Gal4* has no longer any effect on total body weight. (B) Overexpression of the different insertions of the UAS-*l.IMPL2* and UAS-*s.IMPL2* constructs in the fat body by ppl-*Gal4*.

For both, UAS-*l.Imp-L2* and UAS-*s.IMPL2*, exist additional insertions, namely UAS-*l.Imp-L2(*1725*)* and UAS-*s.IMPL2(*1727*)*, that have not been tested yet (Galic 2001). Overexpression of both 1725 as well as 1727 by ppl-*Gal4* resulted in weaker phenotypes than observed with 1724 and 1726 respectively (Fig. S1B). Between the two UAS-*l.IMPL2* transgenic insertions 1724 and 1725 only a narrow difference could be observed, which was even weaker, when the C10-*Gal4* driver was used (Fig. S2). However, a major discrepancy could be observed between the two UAS-*s.IMPL2* transgenic insertions 1726 and 1727 with various Gal4 driver lines (Fig. S1B+S2A). This difference between the two *s.IMPL2* insertions challenges the hypothesis, that overexpressing *s.Imp-L2* has stronger effects than *l.Imp-L2* because it lacks the first exon of *Imp-L2-RB*, and thereby the negative effect on translation of this exon. However, because expression of three different insertions of the UAS-*IMPL2-3T* transgene (Honegger et al. 2006) all showed a stronger inhibition on body weight than UAS-*l.IMPL2* when driven by the C10-*Gal4* driver line (Fig. S2A), we conclude that the weak effect of UAS-*s.IMPL2(1727)* was just due to its unfavorable insertion place.

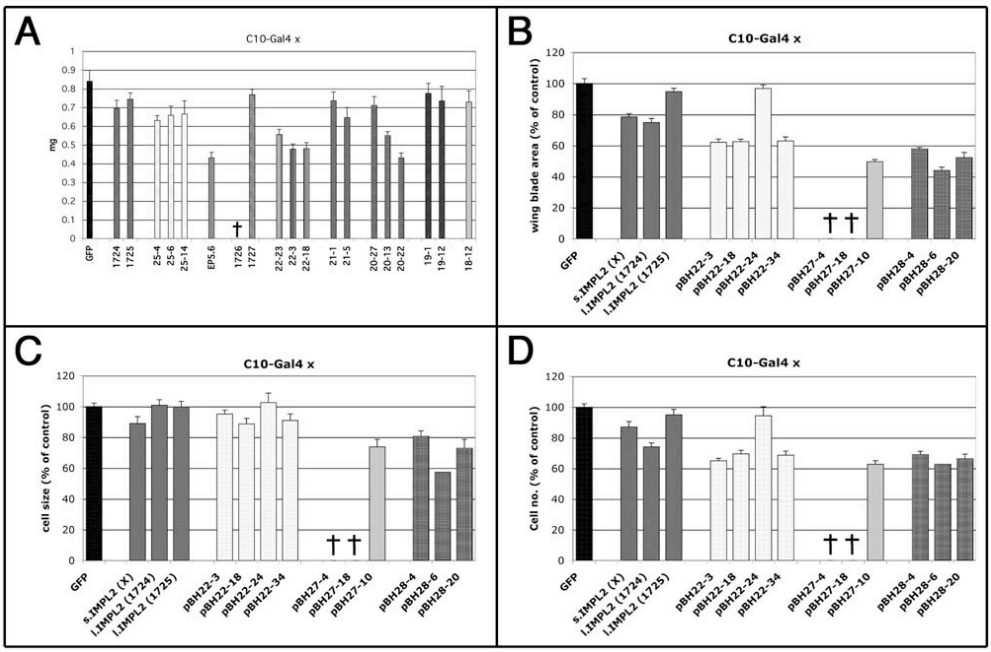


Figure S2: Comparative analysis of the size effects of different UAS-*IMPL2* constructs in the wing.
(A) Weights of 3 day-old adult males reared at 25°C that express different UAS-constructs under the control of the C10-*Gal4* driver line. All flies contain one copy of the driver and one of the UAS-construct. 1724 and 1725 are different insertions of the UAS-*l.IMPL2* transgene. 1726 and 1727 are different insertions of the UAS-*s.IMPL2* transgene. "22-23, 22-3, 22-18" are different insertions of the UAS-*l.IMPL2-3T* transgene. "21-1, 21-5" are different insertions of the UAS-*l.IMPL2* transgene in which the 2nd and the 3rd ATG of the 3 uORFs in exon1 are mutated to TTG. "20-27, 20-13, 20-22" are different insertions of the UAS-*l.IMPL2* transgene in which the 1st and the 3rd ATG of the 3 uORFs in exon1 are mutated to TTG. "19-1, 19-12" are different insertions of the UAS-*l.IMPL2* transgene in which the 1st and the 2nd ATG of the 3 uORFs in exon1 are mutated to TTG. "18-12" is an UAS-*l.IMPL2* transgene in which the 3rd ATG of the 3 uORFs in exon1 are mutated to TTG. (B, C, D) Wing analysis of UAS-*s.IMPL2* (on the X-chromosome), UAS-*l.IMPL2*, UAS-*IMPL2-3T* (pBH22), UAS-*IMPL2-RA* (pBH27), UAS-*IMPL2-RC* (pBH28) driven by C10-*Gal4*. Shown are female wing blade area (B), cell size (C), and cell number (D) as percentage of the C10-*Gal4*, UAS-*GFP* control. Contrary to the females, s.IMPL2(X)-, pBH27-, and pBH28-males did not survive when combined with the C10-*Gal4* driver.

In the process of generating the UAS-*IMPL2-3T* transgene, we also constructed versions of UAS-*l.IMPL2* in which either a single ATG of the three uORFs is mutated to TTG (pBH16, pBH17, pBH18) or two out of three ATGs are mutated (pBH19, pBH20, pBH21). A comparative weight analysis of these different UAS-constructs (except pBH16+17) is shown in (Fig. S2A), where they were driven by the C10-*Gal4* driver displaying low ubiquitous expression and relatively high expression in the developing wing. All the constructs tested showed a decrease in body weight when compared to the GFP control. Though, the extent of the inhibitory effect was variable (see e.g. pBH20 in Fig. S2A).

In addition to the weight measurements, we conducted an analysis about how different UAS-*IMPL2* constructs affect cell size and cell number in the wing. While the size reduction of the two UAS-*l.IMPL2* (1724+1725) and the UAS-*IMPL2-3T* (pBH22) constructs was mainly due to a decrease in cell number (Fig. S2B, C, and D), a third insertion of UAS-*s.IMPL2* (*s.IMPL2(X)*) as well as insertions of UAS-*IMPL2-RA* (pBH27) and UAS-*IMPL2-RC* (pBH28) reduced size by decreasing both cell size and cell number (Fig. S2B, C, and D).

In summary, it is difficult to conclude much about the strength of different transgenes because they are inserted in completely different surroundings in the genome, which greatly influences transcription. Thus, in order to make a clear statement about the differences of the constructs, *Imp-L2* mRNA levels of the various transgenes should be assayed by quantitative real-time PCR (qRT-PCR). This would allow the comparison of insertions with similar expression levels. An alternative would be to generate new transgenic fly lines using the attB/attP-system (Groth et al. 2004), which allows insertion of the transgenes at the very same molecular location, thereby excluding position effects on transgene expression.

Non-autonomous effects of Imp-L2 overexpression

In (Honegger et al. 2006) we showed that overexpressing *Imp-L2* in clones (marked by the absence of the red pigment) decreases cell size in the eye without affecting patterning. Since Imp-L2 is a secreted protein we expected this inhibitory effect on cell size to be non-autonomous. Therefore, by measuring the areas taken up by the photoreceptors (black areas in Fig. S3A), we tried to quantify if the cells in the vicinity of the Imp-L2 source also display altered cell sizes. Even though no significant changes could be observed when one row of photoreceptor clusters was compared with the adjacent one, we saw a tendency to become bigger the farther away from *Imp-L2* clone the clusters are (Fig. S3B).

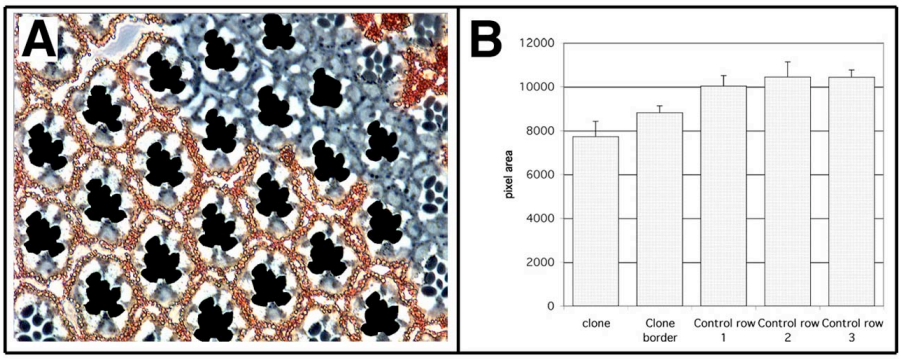


Figure S3: Tissue specific overexpression of *Imp-L2* reduces growth non-autonomously.
(A) Tangential section through an eye containing an *Imp-L2* overexpression clone, which can be recognized by the lack of red pigment. While differentiation and patterning of the *Imp-L2* overexpressing cells appears to be completely normal, cell size is reduced. (B) When the black areas surrounding the photoreceptor cells in (A) is measured, a tendency towards a size increase can be observed the farther away from the clone the photoreceptors are. The clone border was defined as photoreceptor cell clusters that consist of mixed of Imp-L2 overexpressing and control cells. Genotype: (A) *y*, *w*, hs-*flp/y, w*; GMR>*w*+>*Gal4*/+; UAS-*l.IMPL2*/+.

In order to show the non-autonomous effect on size of *Imp-L2* expression in alternative tissue, we expressed it in a wing-specific manner. Overexpression of *Imp-L2* in the posterior compartment by en-*Gal4* not only decreases the size of the posterior wing half, but also of the total wing (Fig. S4A+B). The total wing blade area is reduced by 10% (Fig. S4C). Thus, Imp-L2 expression clearly leads to a non-autonomous decrease in size.

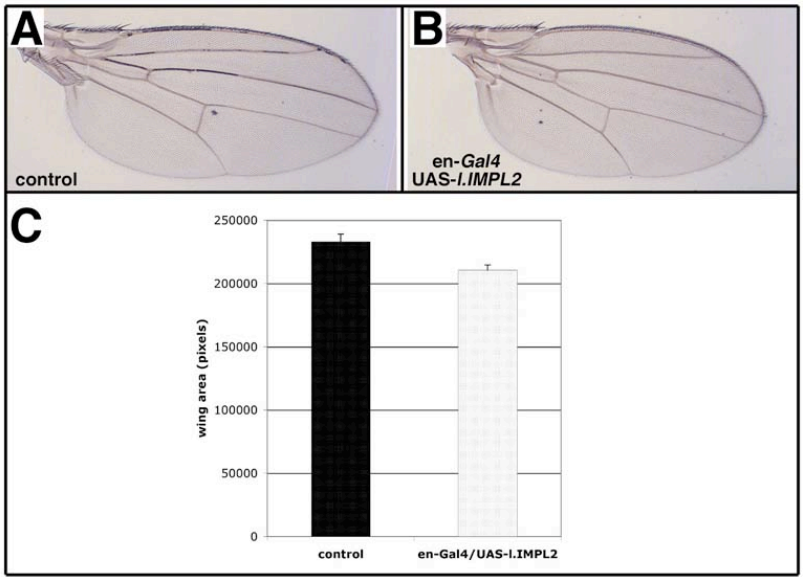


Figure S4: *Imp-L2* decreases wing size non-autonomously when expressed in the posterior compartment by en-*Gal4*.
(A, B) Overexpression of UAS-*l.IMPL2* by en-*Gal4* not only decreases the size of the posterior compartment but of the whole wing blade. Both are right wings from female flies, as control served *y w*- flies. (C) The total wing blade area of en-*Gal4*, UAS-*l.IMPL2* is reduced by 10% compared to the control.

Imp-L2 expression changes metabolism

We have already shown that *Imp-L2* is not essential for survival under standard conditions (Honegger et al. 2006). However, under adverse nutritional conditions, *Imp-L2* is necessary for larvae to survive, and to properly shut down insulin signaling. Next we wanted to assay if *Imp-L2* expression protects against lethality caused by starvation. Therefore, three day-old adult flies were subjected to a water-only diet. Overexpression of *Imp-L2* by ppl-*Gal4* prolongs lifespan of flies under complete starvation (Fig. S5A). Since a resistance to starvation is often caused by elevated body fat levels, we next assayed the fat content of Imp-L2-overexpressing flies. While in *Imp-L2* loss-of-function backgrounds no consistent changes in total fat content could be observed (data not shown), flies overexpressing *l.IMPL2* in the fat body disposed of more fat than controls (Fig. S5B). This is consistent with the fact that *chico* mutants, which display a similar size phenotype as ppl-*Gal4*, UAS-*l.IMPL2* flies, possess double amounts of fat when compared to wild-type flies (Bohni et al. 1999).
Ablating the CC by driving UAS-*rpr* by Akh-*Gal4* results in a strong decrease in the levels of trehalose in the larval hemolymph (Kim and Rulifson 2004). This reduction can be partially rescued by restoring the levels of the functional fly ortholog of glucagon, *dAkh*, which is exclusively produced by the CC cells (Kim and Rulifson 2004). Since Akh only accounts for part of the decrease in circulating trehalose levels, and since dilp2 has been shown to be sufficient to increase sugar levels in the hemolymph (Rulifson et al. 2002), it is tempting to speculate that CC-produced Imp-L2 is, through inhibiting dilp2, able to increase trehalose levels. Therefore, we tested if Imp-L2 is sufficient to increase trehalose levels of the hemolymph. Overexpression of *Imp-L2* in the fat body by ppl-*Gal4* increased trehalose content in the hemolymph of adult females by 12% (Fig. S5C). Contrary to the gain-of-function situation, no consistent changes could be observed in *Imp-L2* mutant combinations.

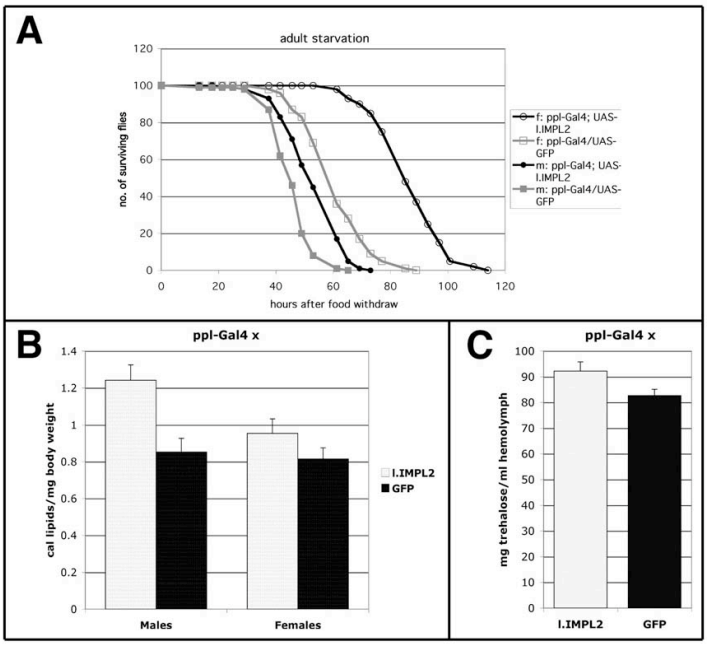


Figure S5: Overexpression of *Imp-L2* in the fat body changes energy metabolism.

(A) *Imp-L2* expression is sufficient to increase survival (males +15%, females +42%) rate under complete starvation in 3 day-old adult flies (of each genotype n=100). Flies were separated after hatching and placed for 3 days on normal food before transferring them to empty vials sealed with a water-soaked foam plug. (B) Overexpression of UAS-*l.IMPL2* in the fat body by ppl-*Gal4* increases the fat content of 3 day-old adult flies (+31% in males, +14% in females). (C) Circulating trehalose and glucose levels are elevated in 3 day-old adult females overexpressing *Imp-L2* in the fat body (males were not assayed).

Additional Imp-L2 loss-of-function data

Comparison of different Imp-L2 alleles

Mutations in the *Imp-L2* gene were generated by two different strategies. First, an EMS-reversion mutagenesis was performed, in which we selected mutant chromosomes carrying EP5.66 that were no longer able to suppress the *dInR* overexpression phenotype in the eye (Honegger et al. 2006). Thereby, one mutant allele was identified, *Imp-L2*^{MG2}, which contained a point mutation in the genomic *Imp-L2* sequence resulting in a premature stop at amino acid 232.

Second, small deletions in the *Imp-L2* locus were obtained by imprecise excision of GE24013 (the line has not been isogenized prior to the screen). Four out of the five deletions obtained, deleted most (Def35) or all (Def20, Def42, Def223) of the *Imp-L2* coding sequence. The fifth

deletion (Def29, not tested) lacks the first exon and part of the following intron of the *Imp-L2-RB* transcript (Honegger et al. 2006). In the process of this jump-out screen we also collected several lines in which GE24013 was precisely excised (named 36, 43, 52). Unfortunately, these precise jump-out lines, as well as the original GE24013 line, contained several point mutations, of which some also resulted in amino acid exchanges in the protein sequence. Therefore, the precise jump-out lines were only used in preliminary experiments as controls.
To analyze the different *Imp-L2* alleles, we assayed the weights of various *Imp-L2* mutant combinations (Fig. S6A+B). Interestingly, $Imp\text{-}L2^{MG2}$ showed no dominant weight increase, which hints at the fact that $Imp\text{-}L2^{MG2}$ is a hypomorphic allele. However, when combined with a null allele ($Imp\text{-}L2^{Def20}$), $Imp\text{-}L2^{MG2}$ displays the same weight increase as $Imp\text{-}L2^{Def223}$ (Fig. S6A). Thus, it was not possible to clearly state if $Imp\text{-}L2^{MG2}$ is a hypomorphic or a null allele, although it behaves rather like a strong hypomorph than a complete null.
To further characterize the *Imp-L2* loss-of-function phenotype, we surveyed the developmental timing of the *Imp-L2* mutants. No differences could be observed between the developmental timing of various *Imp-L2* mutant combinations and the controls (Fig. S6C).

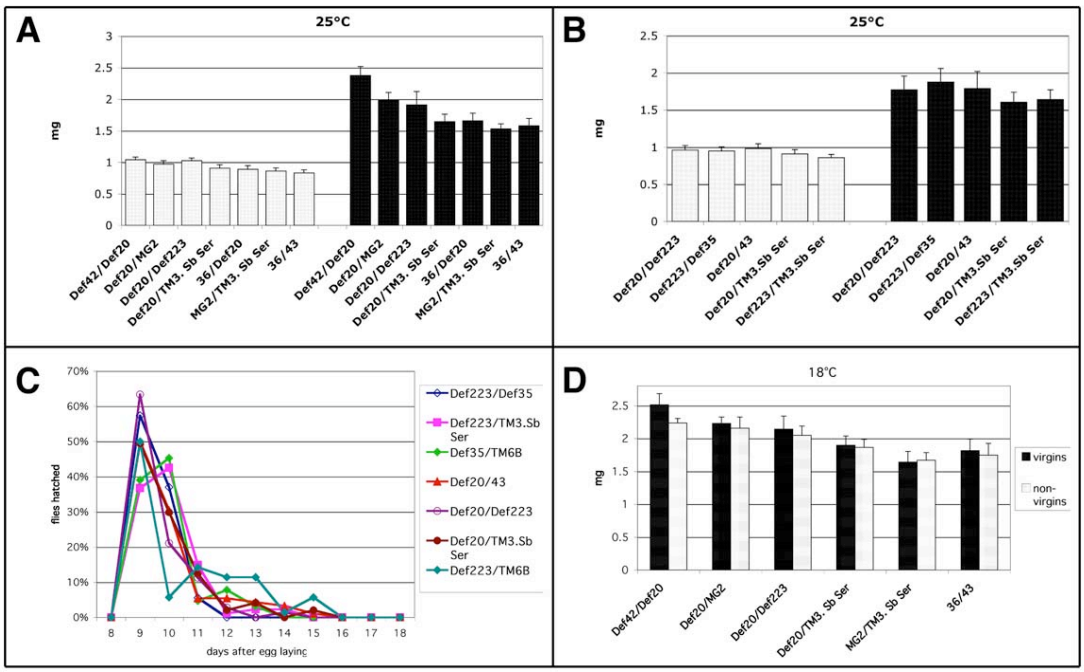


Figure S6: All *Imp-L2* alleles increase total body size without affecting developmental timing.
Different combinations of *Imp-L2* mutant alleles all cause a body weight increase of 3 day-old adult flies (A+B). Flies were reared at 25°C. Males are represented by the white bars, females by the black ones. In a control experiment it could be shown that the TM3 Balancer itself had no effect on total body weight under these conditions (not shown), therefore, it was used as a control. 43 and 36 are precise jump-out alleles of GE24013, containing several point mutations in its *Imp-L2* sequence, some of which resulting in amino acid changes. (C) *Imp-L2* mutants are not developmentally delayed. Of each genotype the hatching flies of two independent tubes were counted. (D) There is no weight difference between 3 day-old virgin and non-virgin females reared at 18°C.

After collecting the hatching flies, they were placed for 3 days in a tube at room-temperature either with (non-virgins) or without (virgins) males. Genotypes: "Def223" is *Imp-L2*^{Def223}, "Def35" is *Imp-L2*^{Def35}, "Def20" is *Imp-L2*^{Def20}, "Def42" is *Imp-L2*^{Def42}, "MG2" is *Imp-L2*^{MG2}, "43" and "36" see above.

Effects of Imp-L2 on oogenesis and oviposition

The relative size increase of *Imp-L2* mutant females is much bigger than of mutant males. This difference is mainly due to massively enlarged ovaries, which consist of more mature eggs and more stage 10 oocytes in *Imp-L2* mutant females, when compared to controls (Honegger et al. 2006). However, the structure of the ovaries is unchanged in *Imp-L2* mutants (Fig. S7A-D). In order to test if the weight increase we observed in mutant females is due an enhanced oocyte production triggered by copulation, we weighed virgin and mated females simultaneously. No difference could be observed between the weights of either control or *Imp-L2* mutant virgins and non-virgins (Fig. S6D). Thus, mating had no effect on the weight of female flies.

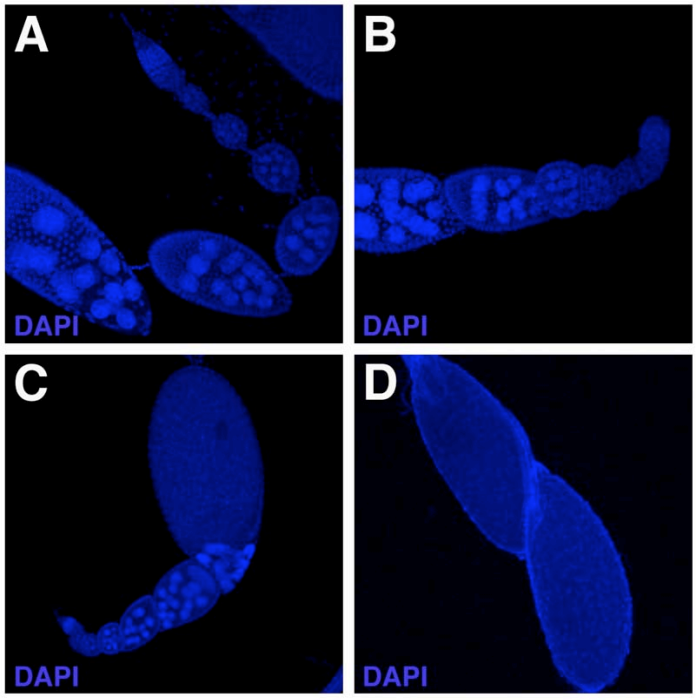


Figure S7: Loss-of-*Imp-L2* function has no obvious effect on the structure of the ovary.
(A-D) Ovaries of *Imp-L2* mutant females, stained with DAPI to highlight the nuclei. Neither in early, intermediate (A-C), nor in late stages of oogenesis (D), a difference in the structure of the ovaries could be observed. Genotype: *y w*;; *Imp-L2*^{Def42}/*Imp-L2*^{Def20}.

Although we could show that the size increase of the ovaries in *Imp-L2* mutants is due to the presence of more mature eggs, it was possible that the accumulation is not solely caused by an increased rate of oogenesis (more stage10 oocytes) but also by a defect in oviposition. Therefore, we assayed oviposition under different nutritional conditions and in different genetic backgrounds. No obvious differences in egg laying could be observed when virgin females were placed together with males on rich food (apple agar plates supplemented with yeast, Fig. S8A). When shifted to poor food conditions (apple agar only), wild-type females downregulate their oogenesis rate by 60-fold and oviposition is blocked (Fig. S8A) (Drummond-Barbosa and Spradling 2001). *Imp-L2* mutants display a lag in shutting down oviposition compared to the control when nutrients become limiting (Fig. S8A). However, after three days on poor food conditions they lay the same amount of eggs as the control. When the flies were placed under poor nutritional conditions right after hatching, neither *Imp-L2* mutants nor heterozygous animals containing a copy of the genomic rescue construct lay more eggs than virgin flies would (Fig. S8B). Also in the shift from poor to rich food, no difference could be observed between *Imp-L2* mutants with or without the rescue construct. Thus, the only difference in oviposition observed was the inability of *Imp-L2* mutants to rapidly block oviposition when shifted from rich to poor food.

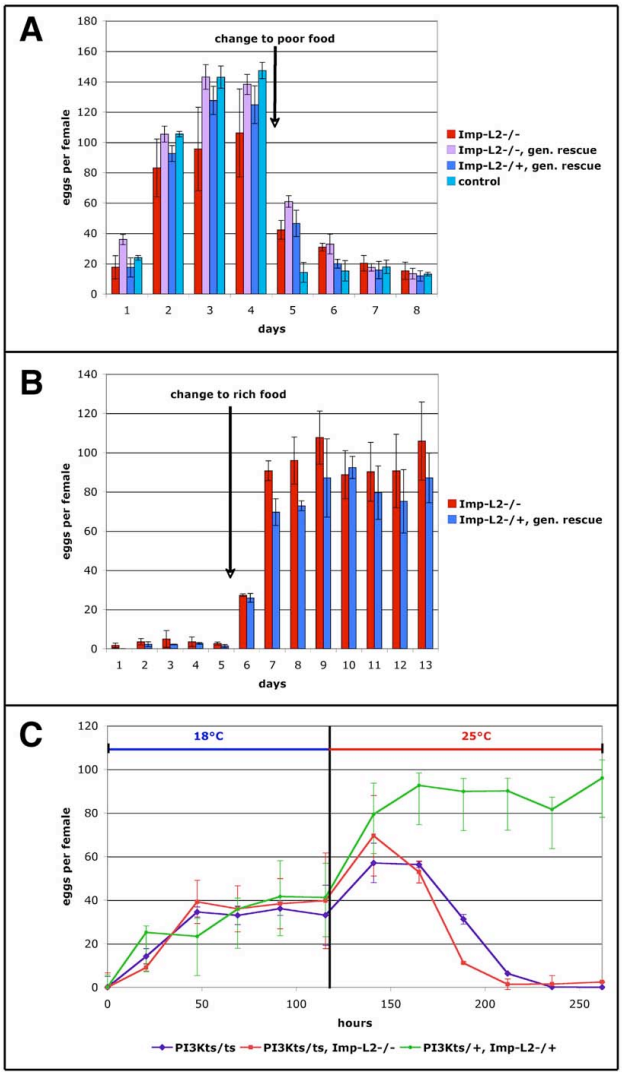


Figure S8: Imp-L2 has no obvious function in the female egg laying behavior

Females (N=15) were collected right after hatching and placed in small egg laying cages (5 females each) together with males. Each day the laid eggs were counted. Two different nutritional conditions were used. “Poor food” confers to just apple agar, whereas “rich food” stands for apple agar supplemented with yeast paste. (A) The egg laying behavior of *Imp-L2* mutants is similar to the controls, except when the food was changed from rich to poor *Imp-L2* mutants were not able to stop egg laying as fast as the wild-type control (compare

red bar of day 5 with the light blue bar). The experiment was repeated twice. (B) When nutrients are limiting *Imp-L2* mutants and controls lay similar amounts of eggs. There was also no difference observed, when the food conditions were changed from "poor" to "rich". (C) Loss-of-*Imp-L2* function is not sufficient to rescue the block in oogenesis observed when PI3K signaling is stalled. $PI3K^{ts}$ (also named $5W3^{iso}$) is a temperature sensitive mutant that displays nearly wild-type PI3K activity at 18°C, but at 25°C displays the characteristics of a strong hypomorphic *PI3K* allele. Genotypes are: "$Imp\text{-}L2^{-/-}$" *y w*;; $Imp\text{-}L2^{Def42}/Imp\text{-}L2^{Def20}$, "$Imp\text{-}L2^{-/-}$, *gen. rescue*" *y w*;; $Imp\text{-}L2^{Def20}$, *pBH5*-57/Imp-$L2^{Def42}$, "$Imp\text{-}L2^{-/+}$, *gen. rescue*" *y w*;; $Imp\text{-}L2^{Def20}$, *pBH5*-57/+, "control" *y w*/*w*, "$PI3K^{ts/ts}$" *y w*;; $5W3^{iso}/5W3^{iso}$, "$PI3K^{ts/ts}$, $Imp\text{-}L2^{-/-}$" *y w*;; $5W3^{iso}$, Imp-$L2^{Def42}/5W3^{iso}$, $Imp\text{-}L2^{Def20}$, "$PI3K^{ts/+}$, $Imp\text{-}L2^{-/+}$" *y w*;; $5W3^{iso}$, $Imp\text{-}L2^{Def20}$/82-1.

In addition to the nutritional requirements, oogenesis is also mediated by the insulin signaling pathway. Genes encoding for its components, namely *dInR*, *chico*, *dPI3K*, *dPKB*, and *dS6K*, mutate to female sterility and block oogenesis at pre-vitellogenic stages (Chen et al. 1996; Bohni et al. 1999; Montagne et al. 1999; Dietz 2003). Further it has been shown by using a temperature-sensitive allele of *PI3K*, $PI3K^{ts}$ (also named $5W3^{iso}$), that the block in oogenesis caused by the lack of insulin signaling activity is reversible in adults (Dietz 2003). Like in starved females, defects in insulin signaling results in an arrest of oogenesis prior to vitellogenesis. However, it was not surprising, that the activity of the insulin signaling pathway was highest around stage 10 of oogenesis (Fig. S9A+B). Since in *Imp-L2* mutants oogenesis is stimulated, we wanted to test if this stimulation is strong enough to overcome the block in oogenesis caused by loss-of-*PI3K* function. *Imp-L2* mutations were not sufficient to overcome the effects of $dp110^{ts}$ mutants when shifted to the restrictive temperature, though (Fig. S8C). Thus, *PI3K* is epistatic over *Imp-L2*.

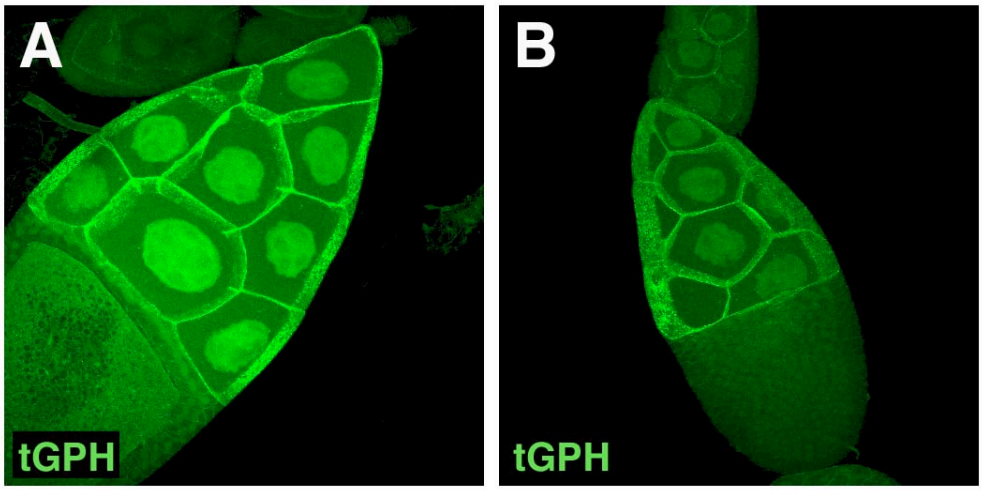


Figure S9: The activity of the insulin signaling pathway in oogenesis peaks during vitellogenesis.
Through localization of the PH-*GFP* (*tGPH*) fusion construct, PIP_3 levels can be monitored. The highest activity of insulin signaling was observed during vitellogenesis. Especially stage 10 oocytes display strong membrane

localization of the *tGPH* reporter (A+B). Shown are projections of a series of confocal pictures. The pictures were taken at the same magnification, and the same exposure time was applied. Shown are fixed samples of 3 day-old female ovaries, with the genotype *y w*; *tGPH*/+.

Stress conditions and *Imp-L2* expression

Effects of starvation on Imp-L2 *expression and mutant survival*

In (Honegger et al. 2006) we showed that loss-of-*Imp-L2* function massively increases starvation sensitivity of 3rd instar larvae. Additionally, Imp-L2 expression was upregulated in the fat body of starved larvae. To assay if also overall levels (including the protein in the hemolymph) of Imp-L2 are increased upon starvation, we extracted protein of starved and non-starved wild-type and *Imp-L2* mutant larvae and compared Imp-L2 protein levels on a Western blot (Fig. S10A+B). Because the loading controls showed different results, no clear statement about a change in Imp-L2 levels could be made in wild-type flies. However, in larvae that were heterozygous for *Imp-L2*, Imp-L2 was upregulated upon starvation in two independent experiments (Fig. S10A+B). Thus, Imp-L2 is required at a certain level when larvae are forced to deal with sparse nutritional conditions.
While *Drosophila* larvae are constant feeders, which take up and process nutrient incessantly, adult flies only feed occasionally. Therefore, a different response of adults and larvae to starvation could be expected. Conversely to *Imp-L2* mutant larvae which have a shortened lifespan under starvation, adults display no increased lethality but live slightly longer than control animals when exposed to complete starvation (water only, Fig. S10C).

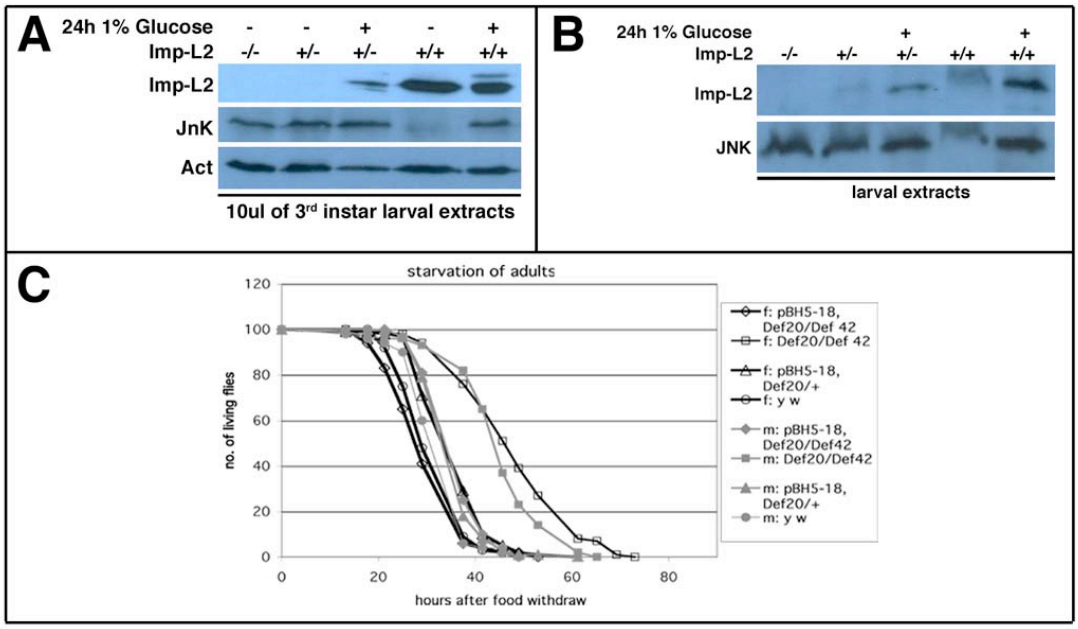


Figure S10: Imp-L2 levels under starvation conditions.

(A+B) 73-76h old larvae of the indicated genotypes were either transferred to a filter paper soaked with PBS containing 1% glucose or an apple agar plate supplemented with yeast for 24h. In both (A+B) 10µl of larval extracts were loaded in each lane. (C) Adult flies (N=100 of each sex per genotype) were collected right after hatching and placed on normal food. After 3 days the flies were separated according to sex and placed in empty culture vials sealed with water-soaked foam stoppers. Every 4-8h the dead flies were counted. For the blots, the following antibodies were used: rat-anti-Imp-L2, rabbit-anti-JnK, mouse-anti-actin. Genotypes: (A+B) "*Imp-L2*$^{-/-}$": *y w*;; *Imp-L2*$^{Def42/Def20}$, "*Imp-L2*$^{+/-}$": *y w*;; *Imp-L2*$^{Def42/+}$, "*Imp-L2*$^{-/-}$": *y w*/*w*, (C) "pBH5-18" genomic rescue construct of *Imp-L2*, "Def20" is the *Imp-L2*^{Def20} allele, "Def42" is the *Imp-L2*^{Def42} allele.

To test if Imp-L2 expression is uprepulated cell-autonomously upon starvation, we downregulated the amino acid transporter *slimfast* (*slif*) specifically in single cell clones of the fat body. For this purpose we made use of a P-element in the *slif* locus (*slif*^{Anti}), that produces, when combined with a Gal4 driver line, *slif* anti-sense RNA (Colombani et al. 2003). This leads to a specific downregulation of *slif* in the Gal4 expressing cell, which causes an amino acid starvation phenotype in the affected cell. Spontaneous fat body clones driving *slif*^{Anti} by Act-*Gal4* did not show a clear upregulation of *Imp-L2* (Fig. S11A, A', A'' and B, B', B''). But since also the expression of Imp-L2 could not be detected when *l.IMPL2* was overexpressed in fat body clones (Fig. S11C, C', C''), either the detection of Imp-L2 in the fat body is difficult under these conditions or the protein is rapidly secreted.

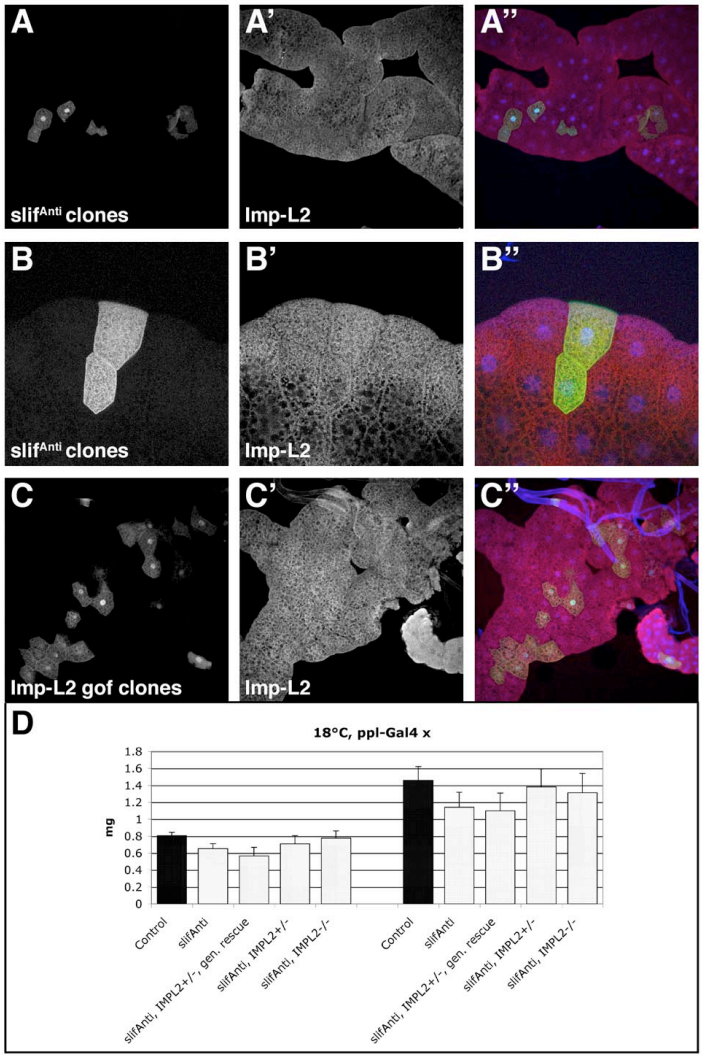


Figure S11: The size decrease resulting from amino acid depletion can be partially overcome in an *Imp-L2* loss-of-function background.

(A, B) Spontaneous clones (marked by GFP) in the fat body of wandering 3rd instar larvae overexpressing the amino acid transporter *slif* anti-sense RNA, produced by a P-element inserted 3' to the *slif* gene. (C) Spontaneous fat body clones overexpressing *Imp-L2* by driving UAS-*l.IMPL2* in the GFP marked cells. (A', B', C') Staining with an Ab against Imp-L2 shows no clear upregulation of Imp-L2 in the *Imp-L2* overexpressing (C') or amino acid-starved cells (A', B'). (A'', B'', C'') GFP in green, Imp-L2 in red. (D) Loss-of-*Imp-L2* function partially rescues the size decrease caused by a downregulation of slif in the fat body induced by ppl-*Gal4* driven $slif^{Anti}$. Shown are weights of 3 day-old adult flies, males to the left, females to the right. Genotypes: (A, A', A'', B, B', B'') *y w*, hs-*flp*; Act>*CD2*>*Gal4*; $slif^{Anti}$, (C, C', C'') *y w*, hs-*flp*; Act>*CD2*>*Gal4*; UAS-*l.IMPL2*, (D) from left to right: *y w*; ppl-*Gal4*/TM3. *Sb*, *Ser*, *y w*; ppl-*Gal4*; $slif^{Anti}$, *y w*; ppl-*Gal4*; $slif^{Anti}$, $IMPL2^{MG2}$/*genomic rescue (57)*, *y w*; ppl-*Gal4*; $slif^{Anti}$, $IMPL2^{MG2}$, *y w*; ppl-*Gal4*; $slif^{Anti}$, $IMPL2^{MG2}$/$IMPL2^{MG2}$.

Downregulating *slif* not solely in single cells but in the whole fat body causes a global growth defect similar to that seen in flies raised under poor nutritional conditions (Colombani et al. 2003). Ppl-*Gal4*, $slif^{Anti}$ flies reared at 25°C mostly die during the pupal stage, but some escapers did hatch that are small and lean. At 18°C most flies hatch but are still slightly smaller than the control animals. It has been shown that this size reduction of ppl-*Gal4*, $slif^{Anti}$ flies is due to a humoral mechanism by which the fat body controls overall body size. Therefore, we speculated if Imp-L2 could be a fat body induced humoral signal controlling total body size by inhibiting Dilp2 activity. Indeed, driving $slif^{Anti}$ by ppl-*Gal4* in an *Imp-L2* mutant background ($Imp\text{-}L2^{MG2/MG2}$) slightly ameliorates the size decrease (Fig. S11D). However, the experiment should be repeated with the small *Imp-L2* deletions to verify this

result. To assess if Imp-L2 is the humoral signal emanating from the fat body, we assayed insulin signaling activity in the epidermis of wild-type or *Imp-L2* mutant wandering 3rd instar larvae, in which *slif* was downregulated specifically in the fat body. Because the signal intensity of the tGPH reporter displayed a great amount of variability (Fig S12), we were neither able to reproduce the published downregulation of tGPH in the periphery of ppl-*Gal4*, $slif^{Anti}$ 3rd instar larvae (Colombani et al. 2003), nor to answer the question if this downregulation is *Imp-L2* dependent. The observed variability was presumably caused by the fact that it was not possible to determine if the used wandering 3rd instar larvae already ceased feeding or not. Depending on the food uptake, the insulin signaling activity varies a lot. Therefore, the experiment should be repeated with early 3rd instar larvae which are constantly feeding.

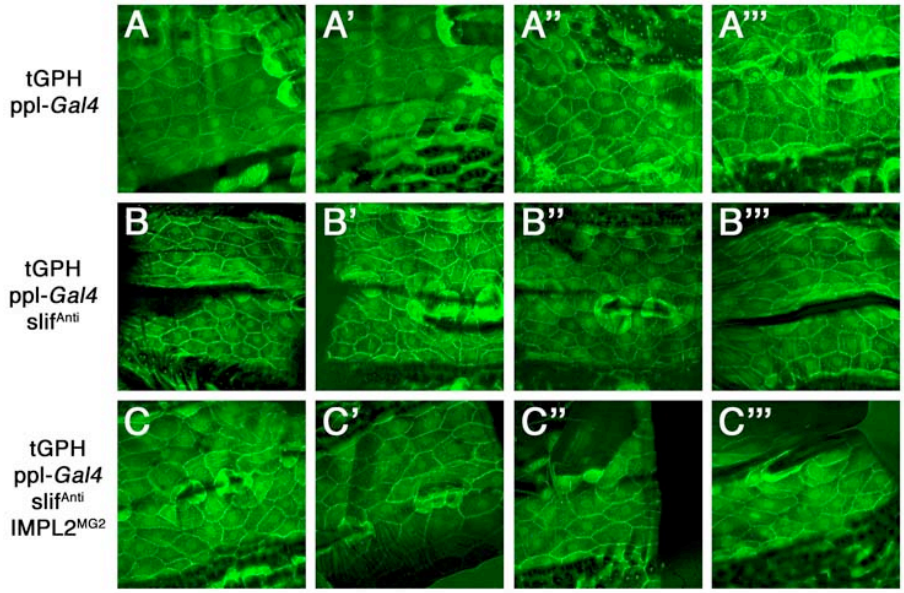


Figure S12: The downregulation of insulin signaling in the periphery induced by fat body starvation could not be reproduced.

(A-C) Shown are tGPH reporter stainings of the epidermis of four independent living 3rd instar wandering larvae of each genotype. Driving the $slif^{Anti}$ P-element in the fat body by ppl-*Gal4* produces *slif* anti-sense RNA. Genotypes: (A) *y w*; tGPH/ppl-*Gal4*, (B)) *y w*; tGPH/ppl-*Gal4*; $slif^{Anti}$, (C) *y w*; tGPH/ppl-*Gal4*; $slif^{Anti}/IMPL2^{MG2}$.

Effects of hypoxia on Imp-L2 expression

Since Imp-L2 is a starvation induced inhibitor of insulin signaling, we wanted to test if it also acts as a general response to stress conditions that require a downregulation of Dilp2 activity. Recently it has been shown in a microarray experiment that *Imp-L2* mRNA is upregulated 4.39 fold when flies are exposed to severe hypoxia (0.5%O_2) for 6h (Liu et al. 2006). By immunohistochemistry we could show that it is again the fat body and no other tissue that induces Imp-L2 expression under hypoxia (Fig. S13, compare A, A', A'' with B, B', B''). Similar to starved larvae, Imp-L2 expression after hypoxic treatment could be detected in small vesicles unequally distributed among fat body cells. Thus, Imp-L2 is not only expressed under starvation conditions but is presumably also needed as an inhibitor of insulin signaling when oxygen becomes limiting.

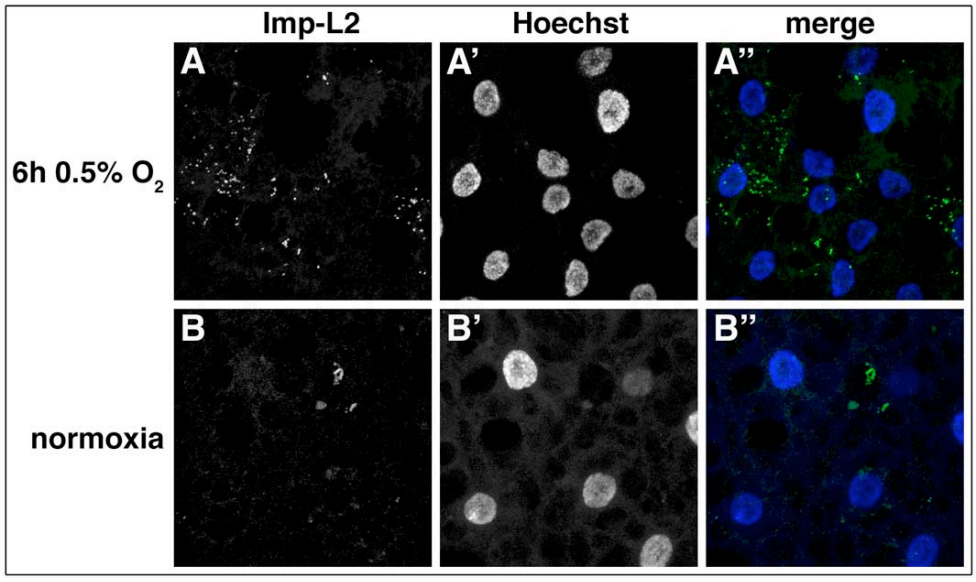


Figure S13: Under hypoxic conditions, Imp-L2 is upregulated by the larval fat body.
73-76h old *y w* 3rd instar larvae were either placed in a hypoxia chamber for 6h at 0.5% O_2 (A, A', A'') or kept under normoxic conditions as controls (B, B', B''). Imp-L2 could be detected in vesicular-like compartments in the cytoplasm of fat body cells. The control fat body, at the same exposure, showed no such Imp-L2-vesicles. The grainy structures in the control were be also observed in *Imp-L2* null mutant larvae.

Genetics interactions of *Imp-L2*

Interaction of Imp-L2 *and* chico

We stated in our paper (Honegger et al. 2006) that loss-of-*Imp-L2* function is not sufficient to rescue the size effect of *chico* mutants (Fig. S14A). Since mild *dilp2* overexpression results in a similar size increase as *Imp-L2* loss-of-function, we also wanted to test if expression of *dilp2* by arm-*Gal4* is able to overcome the size reduction of *chico* mutants. Equally to *Imp-L2* mutants, also in an arm-*Gal4*, UAS-*dilp2* background the size of *chico* mutants is not altered (Fig. S14B). Thus, *chico* is epistatic over *dilp2* as well as *Imp-L2*.

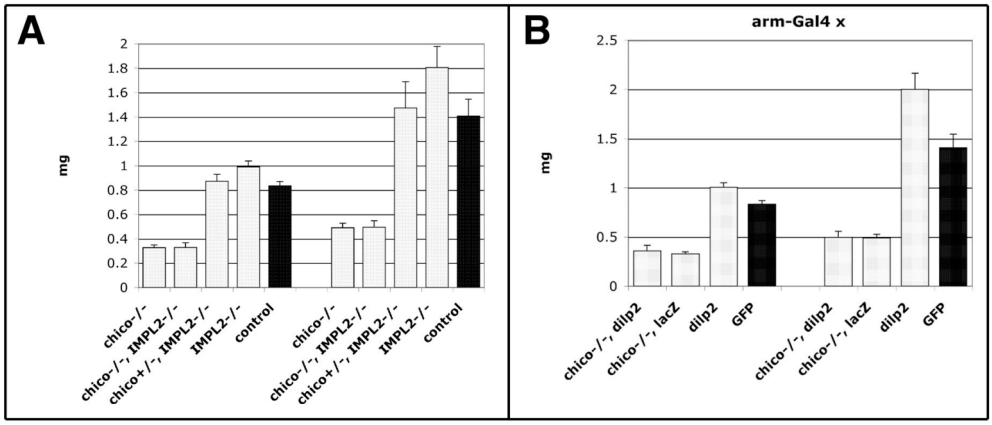


Figure S14: Imp-L2 acts upstream of the insulin signaling pathway

Shown are total body weights of 3 day-old adult flies. The left group within each chart are males and the group on the right side are females. (A) Loss-of-*Imp-L2* function was not sufficient to rescue the size effect of *chico* mutants. In accordance with that, *chico* dominantly suppress the size increase of *Imp-L2* mutants (compare bars 3 and 4, respectively 8 and 9, $p<0.00001$). (B) Weak overexpression of *dilp2* by arm-*Gal4* is also not able to rescue the size reduction of *chico* mutant flies. Genotypes in (A) are from left to right: *y w*; $chico^{2}/chico^{1}$; UAS-*lacZ*/arm-*Gal4*, *y w*; $chico^{2}/chico^{1}$; $Imp\text{-}L2^{Def42}/Imp\text{-}L2^{Def20}$, *y w*; $chico^{2}$/CyO.y^{+}; $Imp\text{-}L2^{Def42}/Imp\text{-}L2^{Def20}$, *y w*;; $Imp\text{-}L2^{Def42}/Imp\text{-}L2^{Def20}$, *y w*; arm-*Gal4*/UAS-*GFP*. Genotypes in (B) are from left to right: *y w*; $chico^{2}/chico^{1}$; UAS-*dilp2*/arm-*Gal4*, *y w*; $chico^{2}/chico^{1}$; UAS-*lacZ*/arm-*Gal4*, *y w*;; UAS-*dilp2*/arm-*Gal4*, *y w*; arm-*Gal4*/UAS-*GFP*.

Interaction of Imp-L2 and the dilps

We described in our paper the finding, that the deficiency Df(3L)AC1, which uncovers *dilp1-5*, dominantly enhanced the small eye phenotype caused by eye specific overexpression of *Imp-L2* (Fig. S15). Since flies heterozygous for Df(3L)AC1 are already slightly reduced in size, it was important to compare the size decrease between the wild-type situation (Fig. S15C) and the heterozygous Df(3L)AC1 situation (Fig. S15D). This size decrease is enhanced when *Imp-L2* is ectopically expressed in the eye (compare Fig. S15A+B with C+D).

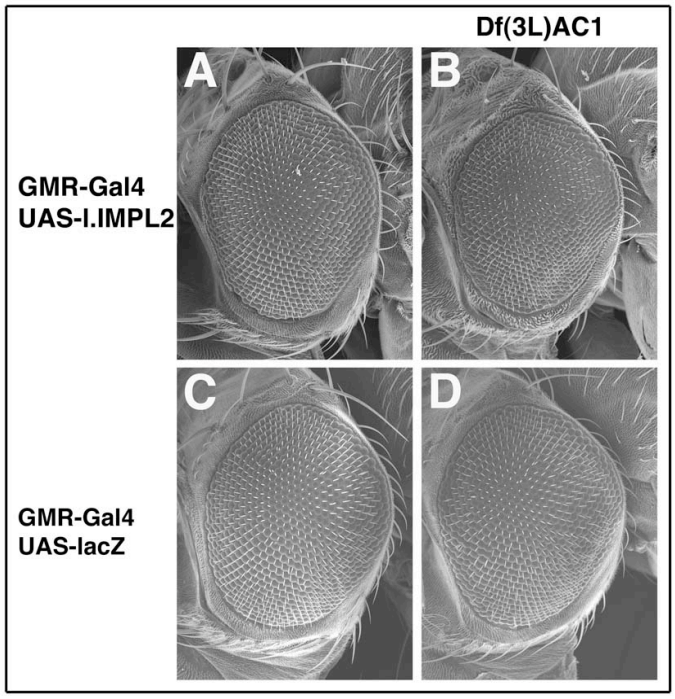


Figure S15: Heterozygosity for *dilp1-5* dominantly enhances the *Imp-L2* overexpression phenotype.
(A) A deficiency uncovering *dilp1-5* (Df(3L)AC1) is dominantly enhancing the reduced eye size of GMR-*Gal4*, UAS-*l.IMPL2* flies. Shown are SEM pictures of female eyes taken with the same magnification.

We further showed in our paper that expression of *dilp2* by arm-*Gal4* in an *Imp-L2* mutant background results in lethality (see also Fig. S16). To assay if Imp-L2 also regulates the function of any of the other Dilp or if it is Dilp2-specific, we tested all the available UAS-*dilp* insertions in the same assay. None of the other *dilps* showed an interaction with *Imp-L2* when expressed under the control of the arm-*Gal4* driver line (Fig. S17A). Since *dilp2* was in our hands the only *dilp* that showed a clear size increase when driven by arm-*Gal4*, we wanted to test if a putative size increase generated by a stronger driver line like i.e. Act-*Gal4*, could be used for a detailed interaction study. Act-*Gal4*, UAS-*dilp2* flies are lethal at larval stages. Conversely, *dilp1*, *3*, *4*, and *7* showed no effect at all on total body size if driven by Act-*Gal4* (Fig. S17B). However, *dilp5+6* displayed a size increase when combined with the Act-driver line. Therefore, we wanted to test the effects of strong *dilp5* or *6* expression in an *Imp-L2*

loss-of-function background. Driving either *dilp5+6* by Act-*Gal4* results in lethality in an *Imp-L2* mutant background (Fig. S17C, from the weaker insertion of *dilp6* - UAS-*dilp6(4)* - two escapers hatched). While overexpressing *dilp5* by Act-*Gal4* had no effect on size in a heterozygous *Imp-L2* mutant background, overexpressing the stronger line of *dilp6* slightly increases total body size in *Imp-L2*$^{+/-}$ flies. Thus, *Imp-L2* seems to interact at least with *dilp2*, *5*, and *6*. Due to the lack of a clear overexpression phenotype for all the other *dilps*, an interaction with *Imp-L2* could neither be excluded nor confirmed.

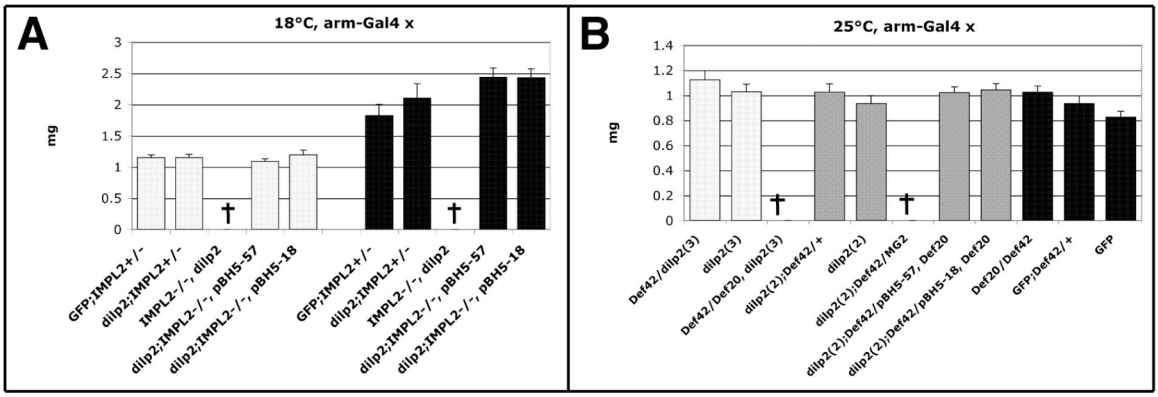


Figure S16: Elevating *dilp2* levels in an *Imp-L2* loss-of-function background results in lethality.
Body weights of 3 day-old adult flies reared either on 18°C (A) or 25°C (B). (A) White bars correspond to the weights of males, black bars to females respectively. All flies contained a copy of the arm-*Gal4* transgene. pBH5-57 and pBH5-18 are two independent insertions of the *Imp-L2* genomic rescue construct. (B) Overexpression of both UAS-*dilp2* lines, on the 2nd (white) and the 3rd (grey) chromosome, by arm-*Gal4* results in lethality only in an *Imp-L2* mutant background. Shown are only male weights.
Genotypes are in (A) from left to right: *y w*; arm-*Gal4*/UAS-*GFP*; *Imp-L2*^{Def42}/+, *y w*; arm-*Gal4*/UAS-*dilp2*; *Imp-L2*^{Def42}/+, *y w*; arm-*Gal4*/+; *Imp-L2*^{Def20}, UAS-*dilp2*/*Imp-L2*^{Def42}, *y w*; arm-*Gal4*/UAS-*dilp2*; *Imp-L2*^{Def20}, pBH5-57/*Imp-L2*^{Def42}, *y w*; arm-*Gal4*/UAS-*dilp2*; *Imp-L2*^{Def20}, pBH5-18/*Imp-L2*^{Def42}, (B) from left to right: *y w*; arm-*Gal4*/+; *Imp-L2*^{Def42}/UAS-*dilp2*, *y w*; arm-*Gal4*/+; *Imp-L2*^{Def20}, UAS-*dilp2*/*Imp-L2*^{Def42}, *y w*; arm-*Gal4*/UAS-*dilp2*; *Imp-L2*^{Def42}/+, *y w*; arm-*Gal4*/UAS-*dilp2*, *y w*; arm-*Gal4*/UAS-*dilp2*; *Imp-L2*^{Def42}/*Imp-L2*^{MG2}, *y w*; arm-*Gal4*/UAS-*dilp2*; *Imp-L2*^{Def20}, pBH5-57/*Imp-L2*^{Def42}, *y w*; arm-*Gal4*/UAS-*dilp2*; *Imp-L2*^{Def20}, pBH5-18/*Imp-L2*^{Def42}, *y w*;; *Imp-L2*^{Def20}/*Imp-L2*^{Def42}, *y w*; arm-*Gal4*/UAS-*GFP*; *Imp-L2*^{Def42}/+, *y w*; arm-*Gal4*/UAS-*GFP*.

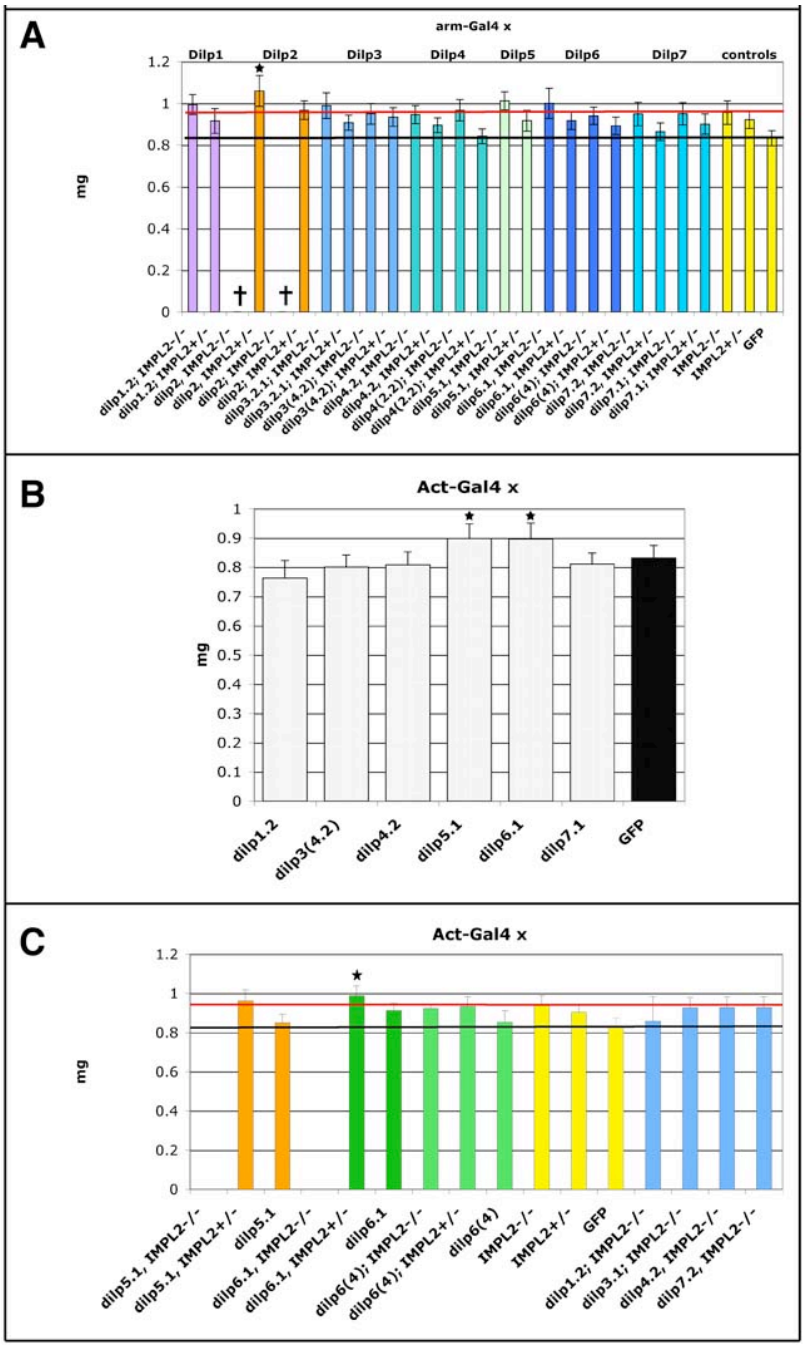


Figure S17: Interaction of the different *dilps* with *Imp-L2*.
(A) Weights of male flies expressing all available *dilp* transgenes by arm-*Gal4* either in a hetero- or homozygous *Imp-L2* mutant background. Each color represents a different *dilp* construct. The red line corresponds to the average weight of *Imp-L2* homozygous mutants, while the black line corresponds to the weight of wild-type flies. Both *dilp2* transgenes result in lethality when combined with arm-*Gal4* in an *Imp-L2* loss-of-function background. (B) Only in the case of *dilp5+6* high levels of *dilp* expression, induced by Act-*Gal4*, led to heavier flies. All the other *dilp* transgenes display no size difference compared to the GFP control. (C) High levels of either *dilp5* or *6* also results in lethality in an *Imp-L2* mutant background. In the case of *dilp5*, the overexpression by Act-*Gal4* is nearly lethal in an *Imp-L2*$^{+/-}$ background. However, several escapers do hatch. The stronger insertion of *dilp6* (UAS-*dilp6.1*) was totally lethal when driven by Act-*Gal4* in an *Imp-L2* loss-of-function situation, while in the case of UAS-*dilp6(4)* two escapers hatched. Flies in which *dilp1* is expressed by Act-*Gal4* in an *Imp-L2* loss-of function background, are semi-viable and vary greatly in size. The overexpression of *dilp3*, *4*, and *7* by Act-*Gal4* was completely viable in *Imp-L2*$^{-/-}$ flies.
Shown are total body weights of 3 day-old male adults. *Imp-L2*$^{-/-}$ always corresponds to *Imp-L2*$^{Def42/Def20}$ (except in the 5th column of chart A, where *Imp-L2*$^{Def42/MG2}$ was used as *Imp-L2*$^{-/-}$. The crosses in C were repeated twice, except the ones with *dilp1*, *3*, *4*, and *7*. (*p<0.0001, in A+C are compared to *Imp-L2*$^{-/-}$, whereas in B the comparison was made against the GFP control).

Interaction of Imp-L2 *and* dALS

Recently an ortholog of the glycoprotein ALS was identified in *Drosophila* (*dALS*) (Colombani et al. 2003). *dALS* is expressed only in the fat body and the *dilp*-producing m-NSCs, and like in mammals its expression is sensitive to starvation (Colombani et al. 2003). In vertebrates, ALS acts as a binding partner of IGF/IGFBP complexes in the vascular system, whereby the half-life of the complex is massively extended (Boisclair et al. 2001). However, the function of ALS in *Drosophila* is unknown. We received several insertions of two unpublished transgenic lines from the Léopold lab in Nice, namely UAS-*dALS* and UAS-*dALS*-RNAi. While the RNAi lines have not been tested yet, we assayed the effects of *dALS* overexpression by different driver lines on total body size in combination with elevated *dilp2* and/or *Imp-L2* levels. Driving UAS-*dALS* by ppl-*Gal4* had no effect on body size of wild-type flies or flies with elevated dilp2 and/or Imp-L2 levels (Fig. S18A). Further, the *Imp-L2* levels produced by ppl>*l.IMPL2* were not high enough, to compensate the hyperinsulinemia induced by overexpressing the weaker UAS-*dilp2*(II) construct by ppl-*Gal4*. In a second experiment, we used the weaker but ubiquitous arm-*Gal4* driver in conjunction with the same overexpression transgenes. While overexpression of *dALS* by arm-*Gal4* in males already led to a significant size increase, it additionally increased the size of flies with higher insulin levels (Fig. S18B). However, this effects could not be observed in females. Therefore, since the effects are rather weak and could only be observed in one sex, the experiment should be repeated before a conclusion can be drawn. In a preliminary experiment, UAS-*dALS* was also driven by the strong Act-*Gal4* driver line. Because driving *dALS* at high levels had no effect on size (Fig. S18C), it is likely that the size increase observed in arm>*dALS* males was only an artifact.

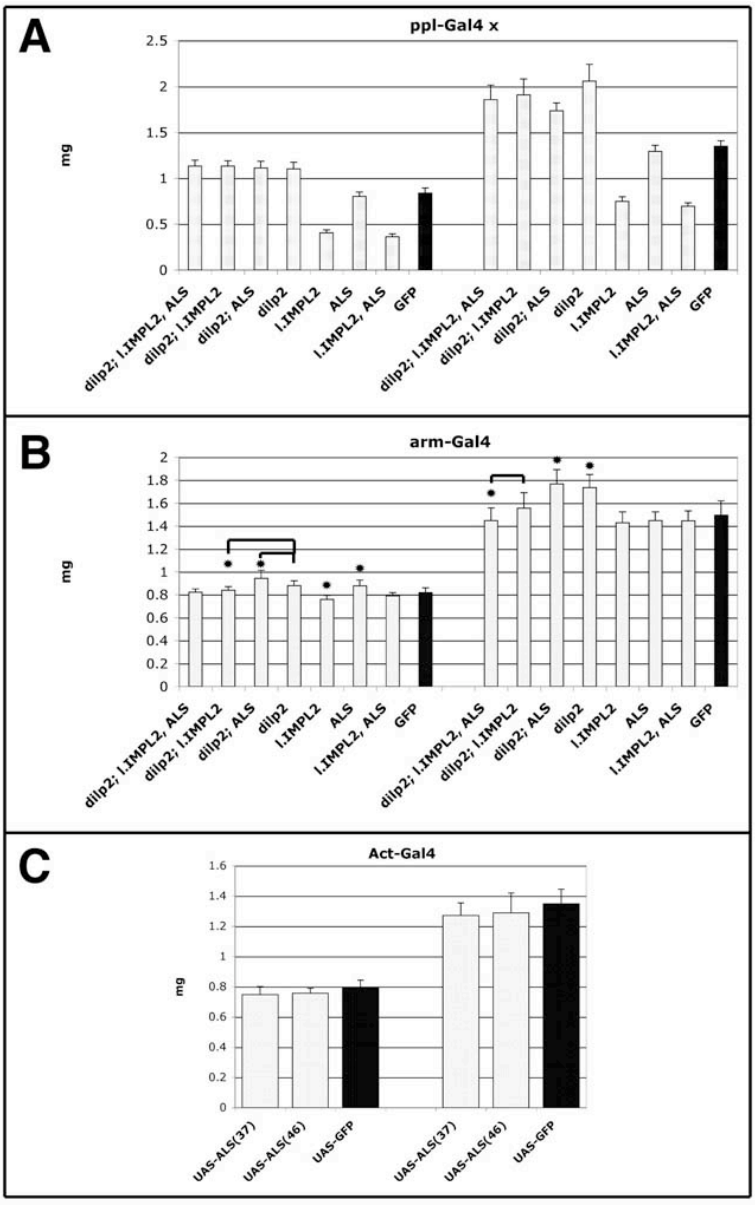


Figure S18: Interaction of *dALS* with *Imp-L2* and *dilp2*

Shown are total body weights of 3 day-old flies, males are to the left and females' weights to the right. (A) Fat body specific overexpression of UAS-*dALS* alone or in combination with either *Imp-L2* or *dilp2* has no effect on total body size. (B) Expression of UAS-*dALS* by arm-*Gal4* resulted in a significant size increase in males but not in females. Co-expression of *dALS* enhances the *dilp2*-induced size increase and the *Imp-L2*-caused size decrease significantly (*p<0.0001, if not otherwise stated the p-value always referred to the GFP control). (C) Strong overexpression of two different insertions of the UAS-*dALS* transgene by Act-*Gal4* had no impact on size. Genotypes in (A) are: *y w*; ppl-*Gal4*/UAS-*dilp2*; UAS-*l.IMPL2*, UAS-*ALS*/+, *y w*; UAS-*dilp2*/+; UAS-*l.IMPL2*/+, *y w*; ppl-*Gal4*/UAS-*dilp2*; UAS-*l.IMPL2*/+, *y w*; ppl-*Gal4*/UAS-*dilp2*; UAS-*ALS*/+, *y w*; ppl-*Gal4*/UAS-*dilp2*, *y w*; ppl-*Gal4*/+; UAS-*l.IMPL2*/+, *y w*; ppl-*Gal4*/+; UAS-*ALS*/+, *y w*; ppl-*Gal4*/+; UAS-*l.IMPL2*, UAS-*ALS*/+, *y w*; ppl-*Gal4*/UAS-*GFP*, in (B) are: *y w*; arm-*Gal4*/UAS-*dilp2*; UAS-*l.IMPL2*, UAS-*ALS*/+, *y w*; UAS-*dilp2*/+; UAS-*l.IMPL2*/+, *y w*; arm-*Gal4*/UAS-*dilp2*; UAS-*l.IMPL2*/+, *y w*; arm-*Gal4*/UAS-*dilp2*; UAS-*ALS*/+, *y w*; arm-*Gal4*/UAS-*dilp2*, *y w*; arm-*Gal4*/+; UAS-*l.IMPL2*/+, *y w*; arm-*Gal4*/+; UAS-*ALS*/+, *y w*; arm-*Gal4*/+; UAS-*l.IMPL2*, UAS-*ALS*/+, *y w*; arm-*Gal4*/UAS-*GFP*, in (C) are: *y w*;; Act-*Gal4*/UAS-*ALS(37)*, *y w*;; Act-*Gal4*/UAS-*ALS(46)*, *y w*; UAS-*GFP*/+; Act-*Gal4*/+.

Human IGFBP-rP1 *cannot mimic* Imp-L2 *expression*

In order to test whether the size effects of *Imp-L2* overexpression can be mimicked by its human ortholog, the *IGFBP-rP1* cDNA was cloned into an UAS-vector to allow its overexpression. Four independent transgenic insertions (no. 1, 3, 6, and 11) of this construct were tested under the control of the ppl-*Gal4* and the Act-*Gal4* drivers. However, no effect on size or development could be observed (Fig. S19A+B). The flies hatched at the expected mendelian ratio, without displaying any timing defects. The crosses were performed at 18°C and 25°C. Thus, although the negative regulation of insulin activity appears to be conserved between the human IGFBP-rP1 and the fly Imp-L2 (Yamanaka et al. 1997; Honegger et al. 2006), the human protein expressed in *Drosophila* cannot take over Imp-L2 function.

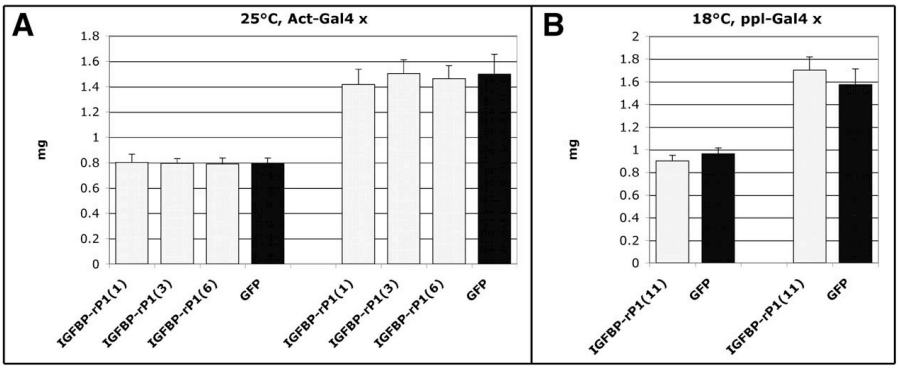


Figure S19: Overexpression of the human *IGFBP-rP1* (*mac25*) is not sufficient to alter growth in *Drosophila*.

Shown are total body weights of 3 day-old adult flies. Males are on the left side of each chart, and females on the right. Driving UAS-*IGFBP-rP1* by either Act-*Gal4* (A) or ppl-*Gal4* (B) had no effect on total body weight. Shown are different insertions of a cDNA derived UAS-transgene, driven by the indicated *Gal4* driver lines.

Is Imp-L2 the "missing link" between Ecdysone and Insulin signaling?

Recently it has been shown that ecdysone (ecd), apart from its role as one of the major hormones regulating development and metamorphosis, antagonizes insulin signaling in determining final body size, without affecting developmental timing (Colombani et al. 2005). Thereby, the larval fat body serves as a key relay element for this ecdysone-dependent growth inhibition. Lowered ecd levels in the hemolymph results in increased insulin signaling in the fat body, and thus in an overall size increase of 17% (Colombani et al. 2005). Since Imp-L2

has been originally identified as ecdysone-inducible protein, and is induced in the fat body upon starvation, it is tempting to speculate that the humoral, insulin-inhibiting signal emanating from the fat body is Imp-L2. We therefore wanted to test if the humoral groth inhibitory effect is blocked in an *Imp-L2* mutant background.

The effects of ecdysone on body size can only be observed when hormone levels are slightly changed. To achieve such a mild alteration in ecdysone levels, the size of the ecdysone-producing prothoracic gland (PG) has to by manipulated by either overexpressing *dp110* or $dp110^{DN}$ (dominant negative version of dp110) by P0206-*Gal4* (Colombani et al. 2005). However, we were only able to reproduce the size increase of P0206-*Gal4*, UAS-$dp110^{DN}$ flies, but not the size decrease of P0206-*Gal4*, UAS-*dp110* flies (Fig. S20A). Our inability to reproduce the results of the Léopold group is probably due to the fact that the food used in Nice and Zurich differs considerably (Arquier et al. 2005; Reiling et al. 2005). Since the yeast content of our food is much higher than in the "Nice"-food the size decrease of P0206-*Gal4*, UAS-*dp110* flies could be masked. Therefore, the experiment should be repeated on food containing low yeast concentrations before Imp-L2 mutations are crossed in.

Since P0206-Gal4 is not only active in the PG but also in the corpora allata (CA), it has to be excluded that the altered insulin signaling in the CA has no effect on final body size. By overexpressing *dp110* and $dp110^{DN}$ with the CA-specific Aug21-*Gal4* driver any effect of the CA on body size in this context could be excluded (Fig. S20B).

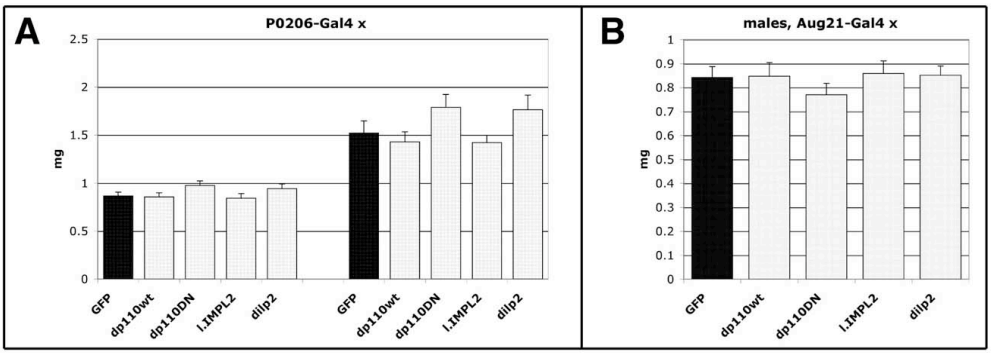


Figure S20: The published size decrease of P0206>*dp110* flies cold not be reproduced

Shown are total body weights of 3 day-old flies. (A) Overexpression of different UAS transgenes in the PG and the CA. While P0206-*Gal4*, UAS-$dp110^{DN}$ flies display the published size increase, P0206-*Gal4*, UAS-$dp110^{wt}$ flies did not show any size difference when compared to the control. Expressing UAS-*l.IMPL2* under the control of P0206-*Gal4* had no effect, but driving *dilp2* led to a significant size increase in both males and females. Males' weights are to the left and females' weights to the right. (B) With the CA specific Aug21-*Gal4* driver, $dp110^{wt}$, *l.IMPL2*, and *dilp2* overexpression had no effect, while Aug21-*Gal4*, UAS-$dp110^{DN}$ displayed a slightly decreased body size. Genotypes are in (A): *w/yw*; P0206-*Gal4*/UAS-*GFP*, *w/yw*; P0206-*Gal4*/UAS-$dp110^{wt}$, *w/yw*; P0206-*Gal4*/+; UAS-$dp110^{DN}$/+, *w/yw*; P0206-*Gal4*/+; UAS-*l.IMPL2*/+, *w/yw*; P0206-*Gal4*/+; UAS-

dilp2/+, in (B): *w*/*yw*; Aug21-*Gal4*/UAS-*GFP*, *w*/*yw*; Aug21-*Gal4*/UAS-*dp110*^{wt}, *w*/*yw*; Aug21-*Gal4*/+; UAS-*dp110*^{DN}/+, *w*/*yw*; Aug21-*Gal4*/+; UAS-*l.IMPL2*/+, *w*/*yw*; Aug21-*Gal4*/+; UAS-*dilp2*/+

Generation and testing of cell culture tools

The UAS-IMPL2-HA *construct*

In order to obtain a tagged version of Imp-L2, which can be used for cell culture and pull-down experiments with sepharose beads, we added to the C-terminus of *s.IMPL2* a 4xHA tag and cloned it into the UAS vector. Because a tag can in some cases destroy the original function of a protein, lead to its degradation or result in a neomorph, we generated transgenic fly lines to test if the tagged version of Imp-L2 is still functional. Ectopic expression of UAS-*IMPL2-HA* posterior to the morphogenetic furrow of the eye imaginal disc by GMR-*Gal4* shows that the tagged version of Imp-L2 could be stably expressed and stained by both antibodies against Imp-L2 and the HA tag (Fig. S21A-C). Further, driving UAS-*IMPL2-HA* either by C10-*Gal4* (Fig. S21D) or ppl-*Gal4* (Fig. S21E) reduced total body size to a similar extent as UAS-*l.IMPL2*. Thus, we conclude that Imp-L2-HA is a functional version of the Imp-L2 protein that did not show enhanced degradation or neomorphic functions.

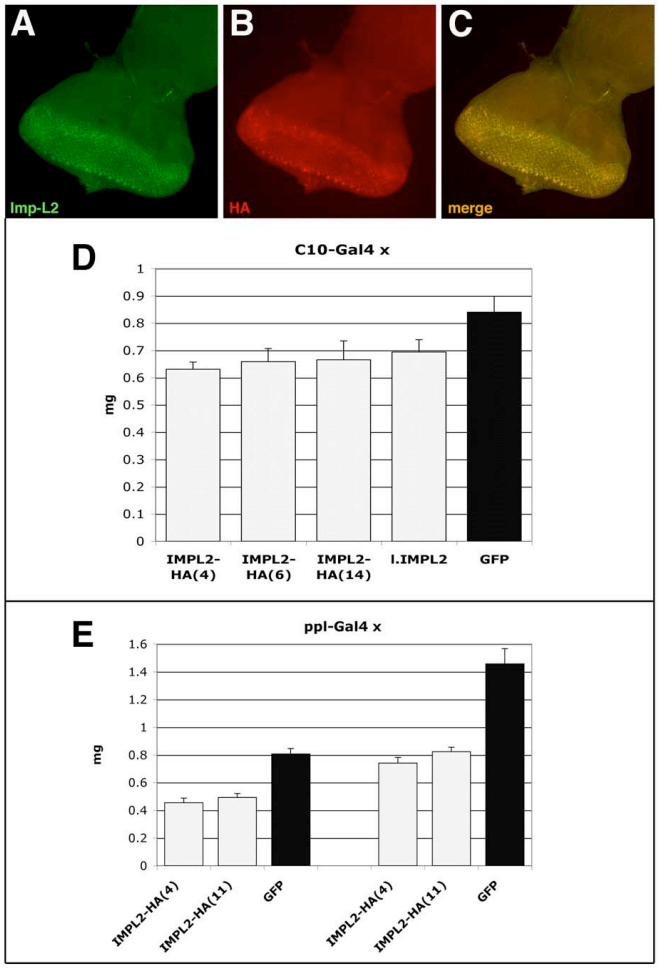


Figure S21: The UAS-*IMPL2-HA* (pBH25) construct is functional.

(A-C) Expression of UAS-*IMPL2-HA*(14) by GMR-Gal4 leads to ectopic expression of Imp-L2 in the developing eye disc posterior to the morphogenetic furrow. This expression of Imp-L2 can be either detected by

an anti-Imp-L2-Ab (A, green) or an Ab against HA (B, red). (D) C10-*Gal4* driven expression of different insertions of the UAS-*IMPL2-HA* results in a decrease in total body size that is comparable to UAS-*l.IMPL2*. Shown are male weights (bar1-3: N=20; bar4+5: N=30). (E) Also expression of UAS-*IMPL2-HA* in the fat body by ppl-*Gal4* results in flies of massively decreased size.

Because Imp-L2 could not be detected in the spontaneous fat body clones with UAS-*l.IMPL2* we wanted to test if the protein could be stained better using the HA antibody and the UAS-*IMPL2-HA* construct. Neither in *IMPL2-HA* overexpression clones of the fat body nor in clones of the salivary gland a clear signal for Imp-L2 could be detected (Fig. S22A, A', A'' and B, B', B''). However, in overexpression clones of the midgut, Imp-L2 expression could be detected with the HA antibody (Fig. S22C, C', C''). Thus, it seems as if the protein expressed in fat body as well as salivary gland cells is rapidly secreted and therefore difficult to detect in overexpression clones.

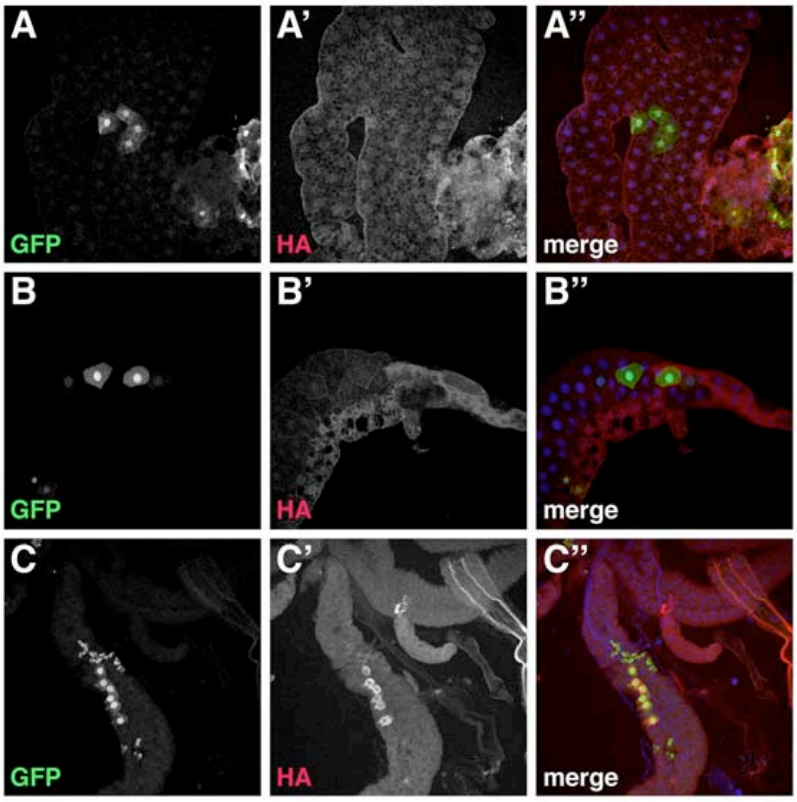


Figure S22: Overexpression of HA-tagged *Imp-L2* was not detectable by immunohistochemistry in fat body and salivary glands but in midgut clones.
(A-C) Spontaneous overexpression clones of UAS-*IMPL2-HA(11)* in different tissues of wandering 3rd instar larvae marked by GFP. Shown are clones in the fat body (A, A', A''), the salivary gland (B', B', B''), and the midgut (C, C', C''). Genotype: *y w*, hs-*flp*; Act>*CD2*>*Gal4*; UAS-*IMPL2-HA(11)*.

The UAS-Flag-dilp2 *construct*

To test if Imp-L2 is physically binding to insulin, we first wanted to assay the best way of detecting insulin on a Western Blot. On an acrylamide gel the band corresponding to the B-chain of human insulin runs below 10kDa (Fig. S23A). The A-chain, which should be around 3kDa in size was not detectable on this sort of gel (16% acrylamide gel). When blotted shortly, the B-chain could be also detected by the human insulin anti-body (Fig. S23B). Next we wanted to know if we could also detect Dilp2 on a Western blot. The rabbit-anti-dilp2 antibody created by P. Belawat of our lab was so far only tested by immunohistochemistry, where it worked nicely. To test if the antibody also works on Western blots we loaded different amounts of recombinant Dilp2 protein (produced by P. Belawat) on an acrylamide gel. Unfortunately, Dilp2 could just be detected as a smear (Fig. S23C), which could not be used for a pull-down assay together with Imp-L2.

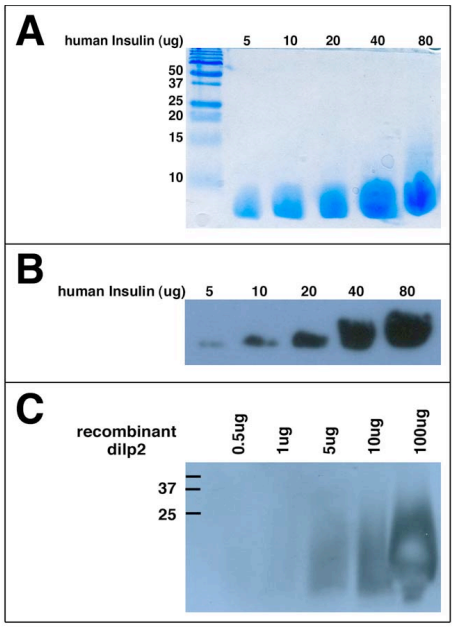


Figure S23: Detection of insulin on a Western blot.
Different amounts of human insulin were loaded on a acrylamide gel and either stained with commassie blue (A) or blotted for 30min with 150mA at 4°C and stained with an Ab against human insulin (B). In (C), different amounts of recombinant dilp2 (P. Belawat) were loaded, blotted like human insulin, and detected by anti-dilp2-Ab. All the bands correspond to the B-chain of the insulins.

Because we could not use the Dilp2 antibody for experiments including Western Blots, we wanted to create a tagged version of Dilp2. In a first attempt T. Ikeya of our lab constructed a version of Dilp2 that contained a C-terminal 4xHA tag. However, the UAS-*dilp2-HA* construct was not functional (transgenic flies did not show a size increase or any other effect when combined with various driver lines). Therefore, in a second attempt we decided to tag Dilp2 N-terminally with a single copy of the short flag tag. We introduced the flag tag after the predicted signal peptide sequence of *dilp2* (Brogiolo et al. 2001), and thereby replaced the original Dilp2 signal peptide by the hemagglutinin signal peptide (Fig. S24A). The *Flag-*

Dilp2 construct was then cloned into an UAS vector. In S2 cells transiently transfected with UAS-*Flag-Dilp2* in conjunction with Act-*Gal4* expression of Flag-Dilp2 could be detected (Fig. S24B). However, why Flag-Dilp2 always appears as a double band is unclear. It ran between 15kDa and 20kDa, which is much bigger than the usual band of the insulin B-chain (compare with Fig. S23). If the detected protein corresponds to unprocessed pro-Dilp2, or if one band is pro-Dilp2 and the other the Flag tagged B-chain (even though it is too big) is unknown. It is also remains elusive if S2 cells are capable of processing Dilp2 correctly. A way of testing if the S2 cells are able to produce functional and active Dilp2, is to stimulate wild-type cells with supernatant of Flag-*dilp2* expressing S2 cells, and monitor PKB and S6K phosphorylation states.

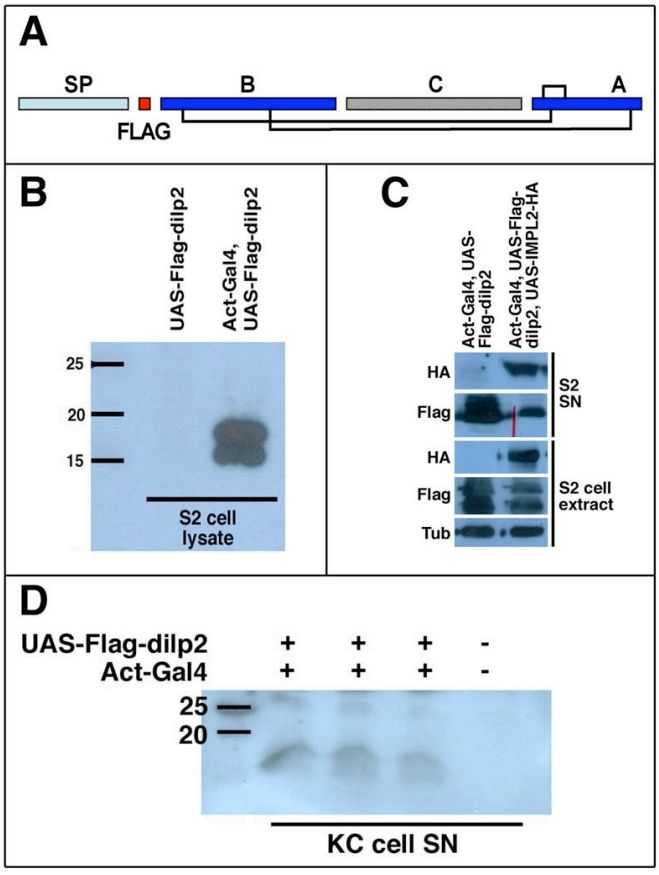


Figure S24: Construction of a Flag-tagged *dilp2* construct.
(A) Overview of the UAS-*Flag-dilp2* construct. The signal sequence of dilp2 was removed and replaced by the hemagglutinin signal peptide (SP) sequence (from the PS261 vector). After the signal sequence, a single copy of the Flag tag was inserted. Thus, the created dilp2 version contains a N-terminal Flag tag. (B) Western Blot of S2 cell extracts that were transiently transfected with either UAS-*Flag-dilp2*, or Act-*Gal4*, UAS-*Flag-dilp2*, and incubated with an anti-Flag antibody. (C) Western of two different stable S2 cell lines, expressing either the Flag-tagged *dilp2* alone or together with the *Imp-L2-HA* construct. The two top lanes arre the supernatants of the stable cell lines, while the three lanes at the bottom are cell lysate. These cell lines were used for the *in vitro* pull-down assay together with the *in vitro*-translated Imp-L2 (Honegger et al. 2006). (D) Western Blot of supernatant from three independent KC cell lines, incubated with anti-Flag antibody.

To verify if the UAS-*Flag-dilp2* produces functional Dilp2, we generated transgenic fly lines. Driving seven different insertions of UAS-*Flag-dilp2* by Act-*Gal4* resulted in all cases in a significant size increase (Fig. S25A). Since UAS-*dilp2* driven by Act-*Gal4* results in lethality, and arm-*Gal4* driven overexpression of UAS-*Flag-dilp2* only with one insertion led to a size

increase (Fig. S25B), we concluded that Flag-Dilp2 is a functional version of Dilp2 with slightly decreased activity compared to the wild-type protein. To test this hypothesis, we wanted to test if the *Flag-dilp2* is also able to interact with *Imp-L2*. Co-overexpression of UAS-*Flag-dilp2* is sufficient to rescue the size decrease induced in Act-*Gal4*, UAS-*IMPL2-HA* flies (Fig. S25C). Thus, Flag-Dilp2 is a functional version of Dilp2 that displays slightly diminished activity.

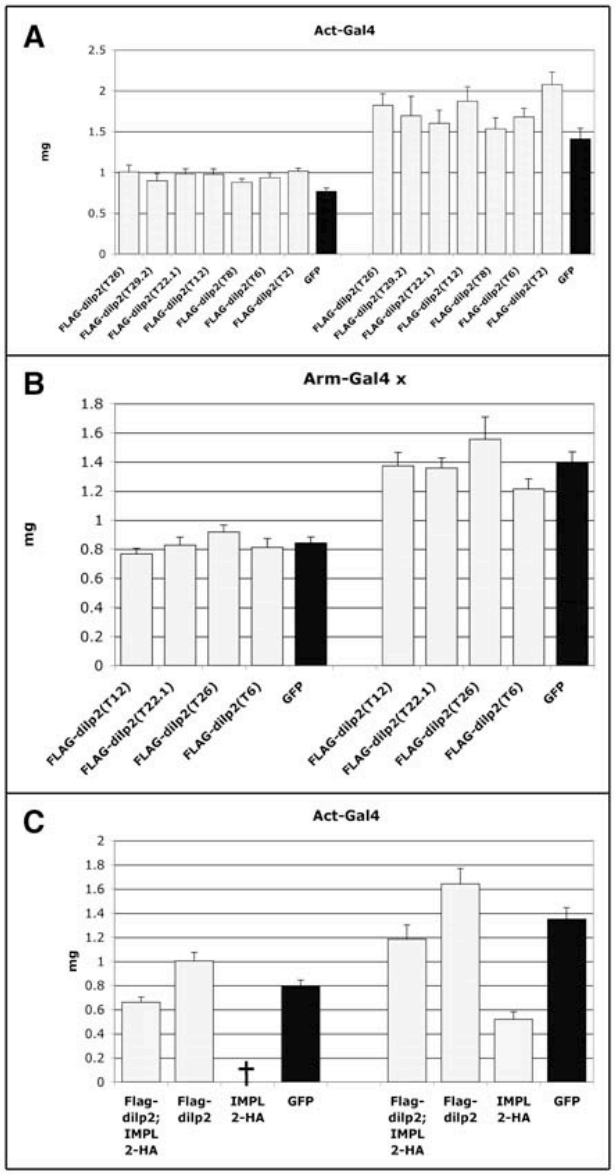


Figure S25: The UAS-*Flag-dilp2* construct is functional but weaker than non-tagged dilp2. (A) Overexpression of seven different insertions of the UAS-*Flag-dilp2* construct by Act5C-*Gal4* each resulted in an increase of total body weight. (B) The weaker arm-*Gal4* driver only increased the size of the strong insertions like UAS-*Flag-dilp2*(T26). (C) Tagged versions of Imp-L2 and dilp2 still counteract each other's effect on size (the experiment was also done with the ppl-*Gal4* driver). Genotypes in (C) are: *y w*; UAS-*Flag-dilp2*(T22.1)/+; UAS-*IMPL2-HA*(pBH25-11)/Act5C-*Gal4*, *y w*; UAS-*Flag-dilp2*(T22.1)/+; Act5C-*Gal4*/+, *y w*;; UAS-*IMPL2-HA*(pBH25-11)/Act5C-*Gal4*, *y w*; UAS-*GFP*/+; Act5C-*Gal4*/+.

Since we could show that Flag-Dilp2 was functional, we next established stable cell lines that express *Flag-dilp2* under the control of Act-*Gal4*. In stable S2 cell lines transfected with either Act-*Gal4*, UAS-*Flag-dilp2* or Act-*Gal4*, UAS-*Flag-dilp2*, UAS-*IMPL2-HA*, both Flag-Dilp2 and Imp-L2-HA could be detected in cellular extracts as well as supernatants (Fig.

S24C). Also in stable KC cell lines Flag tagged Dilp2 protein could be detected either in cell extracts or the supernatant (Fig. S24D).

Stable Imp-L2-HA-*expressing cell lines*

To further study the interaction of Imp-L2 with Dilp2 we wanted to obtain purified Imp-L2 protein. Therefore, we generated several stable cell lines that produce and secrete Imp-L2. Since it was doubtful that cells constantly overexpressing the growth inhibitor *Imp-L2* would grow, if stably transfected, we generated cell lines containing an inducible *Imp-L2* construct. For this purpose we cloned the *Imp-L2-HA* into a pMT vector which contains an inducible metallothionein promotor. The MT-*Imp-L2-HA* construct can be induced by $CuSO_4$. We generated KC as well as clone8 stable cell lines that contained the MT-*Imp-L2-HA* construct. Interestingly, the clone8 cells were more efficient in producing and secreting Imp-L2 (Fig. S26A). Thus, for most of the cell culture experiments, the stable MT-*Imp-L2-HA* clone8 (named Cl8-1) were used.

In addition to the cell lines containing the inducible MT-*Imp-L2-HA* construct, we also generated stable clone8 cell lines that constitutively express *Imp-L2-HA*, containing Act-*Gal4* and UAS-*IMPL2-HA* (Cl8-7, Fig. S26B). Like the Cl8-1 cells, also the Cl8-7 cells grew and were easy to keep.

To conduct a co-immunoprecipitation (co-IP) with Imp-L2 and dilp2, we also generated cell lines expressing both genes. Of the three different transfections, only the Cl8-11 cell line stably expressed Imp-L2 and dilp2, although the expression levels are lower than in cells transfected with only one of the genes (Fig. S26B). However, the IP of Imp-L2 and Dilp2 out of these cell lines never worked.

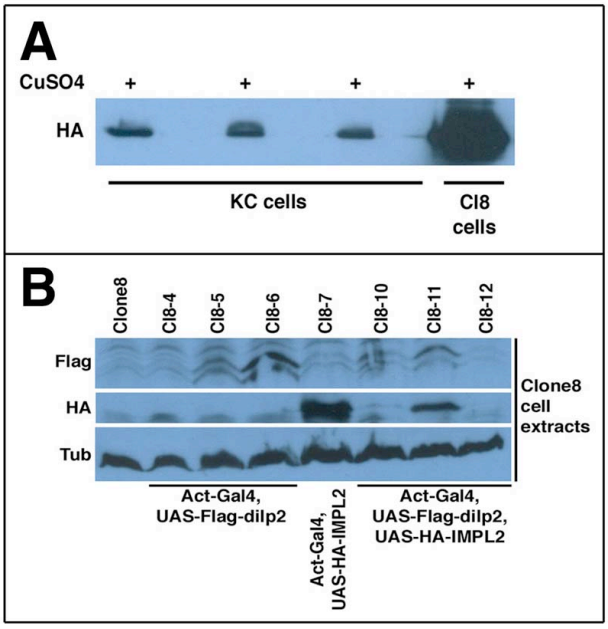


Figure S26: Test of stable *Imp-L2-HA* cell lines. (A) Stable KC cells (except lane 7) expressing MT-*IMPL2-HA* starved overnight in serum- and insulin-free medium (Shields & Sang supplemented with Penicillin/Streptomycin only). Expression of *Imp-L2* was either induced (+ $CuSO_4$) overnight or not. The same amounts of supernatants of each sample was loaded on a gel and blotted. Lanes from left to right: 1+2: KC-??, 3+4: KC-??, 5+6: KC-??, 7: Cl8-1. (B) Test of the stable Clone8 cell lines, which were transfected with the indicated constructs.

Cell culture experiments

Imp-L2 *RNAi in S2 cells*

S2 cell extracts showed, when analyzed on a Western blot, a band of around 30kDa that could be stained with the Imp-L2 antibody. To verify that *Imp-L2* is expressed by S2 cells we treated S2 cells for seven days with double stranded RNA of *Imp-L2* to downregulate its expression. Imp-L2 expression could be efficiently downregulated in two independent experiments by RNAi against it (Fig. S27A+B). This downregulation of Imp-L2 did not change the way the cells reacted upon insulin stimulation. Interestingly, *Rheb* RNAi, which served as a positive control, also slightly downregulated *Imp-L2* expression (Fig. S27A). But since the second RNAi experiment did no longer show such a clear downregulation of Imp-L2 when the cells were treated with RNAi against *Rheb* (Fig. S76B), this result should be further verified by additional RNAi experiments (also against other pathway members) before any conclusions can be made.

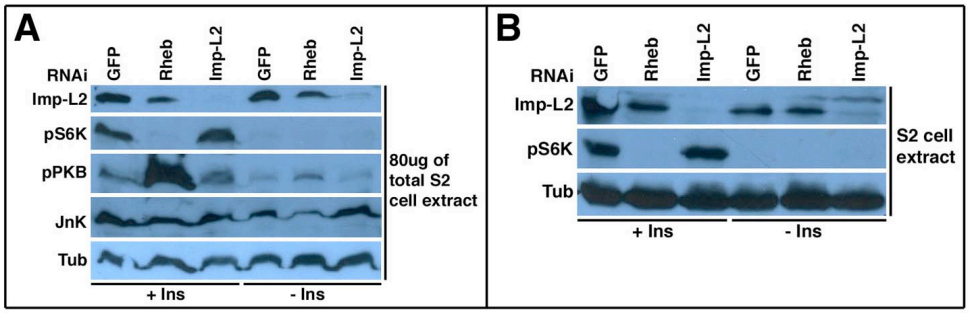


Figure S27: Imp-L2 downregulation does not affect insulin stimulation in S2 cells.
Double-stranded RNAs of the indicated genes were added to S2 cells. After 7 days cells were either stimulated for 30min with bovine Insulin (lanes 1-3) or not (lanes 4-6). (A+B) No difference in phosphorylation of S6K and PKB could be observed between cells containing either high or low levels of Imp-L2 (compare lanes 1 and 3, and 4 and 6 respectively). In cells treated with RNAi against Rheb, Imp-L2 levels were, especially when stimulated with insulin, slightly decreased. The following antibodies were used: rat-anti-Imp-L2, rabbit-anti-phospho-S6K, rabbit-anti-phospho-PKB, rabbit-anti-JnK, mouse-anti-tubulin.

Insulin stimulation in Imp-L2 expressing cells

As we have shown in the manuscript (Honegger et al. 2006), *Imp-L2* expression affects growth by regulating insulin signaling activity in flies. We next wanted to study the effect of Imp-L2 on insulin stimulation in cell culture. In order to optimize the conditions for the insulin stimulation we exposed clone8 cells to various nutritional conditions before stimulating with human insulin. Only when the cells were starved in serum-free medium (supplemented only with penicillin and streptomycin) over night prior to the stimulation, a difference in S6K phosphorylation could be detected between stimulated and non-stimulated cells (Fig. S28). Thus, in further experiments (if not stated otherwise) cells were starved before stimulated with insulin.

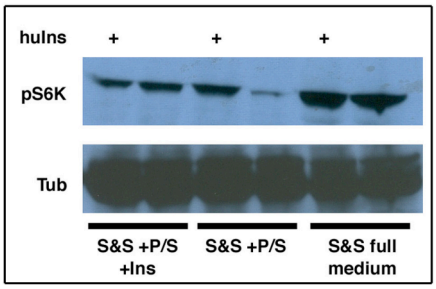


Figure S28: Clone8 cells need to be starved before stimulation.
Shown is a Western blot of different cell extracts from Clone8 cells. The samples loaded in the first two lanes were kept in serum-free culture medium (Shields & Sang supplemented with Insulin and Penicillin/Streptomycin). In lanes 3+4, cells were starved o/n in Shields & Sang supplemented with Penicillin/Streptomycin only. The cells of lane 5+6 were kept in full culture medium (Shields & Sang supplemented with fetal calf serum, Insulin, and Penicillin/Streptomycin). The Blot was stained with an Ab against phosphorylated dS6K and Tubulin as a loading control,.

To assay the stimulation of the insulin pathway in cells, phosphorylation levels of dS6K and dPKB were analyzed after 30min of insulin stimulation. KC cells transiently transfected with Act-*Gal4*, UAS-*IMPL2-HA* could be stimulated to the same extent as control cells by human insulin (Fig. S29A).

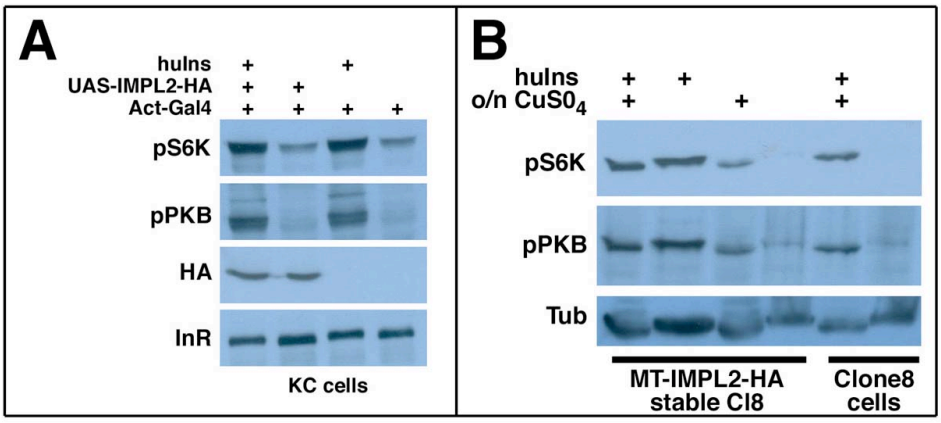


Figure S29: The presence of Imp-L2 had no effect on stimulation with human insulin in cell culture. (A) Transiently transfected KC cells did not display diminished stimulation when treated with human insulin. Cells were either transfected with both Act-*Gal4* and UAS-*IMPL2-HA* or Act-*Gal4* alone. After 2 days cells were stimulated for 30min with huIns. (B) Stable cell lines containing an MT-*Imp-L2-HA* vector were starved o/n in serum- and insulin-free medium. During this time $CuSO_4$ was added to the samples in lane 1, 3, and 5. The cells were then stimulated for 30min with human insulin. The different sizes of the tubulin bands are due to the presence of the $CuSO_4$, which was not washed away totally. "pS6K" refers to an Ab against phosphorylated dS6K, "pPKB" refers to an Ab against phosphorylated dPKB.

Also in stable clone8 cells (Cl8-1, MT-*IMPL2-HA*) the stimulation by human insulin was not altered by the presence of Imp-L2 in the medium (Fig. S29B). However, since it has been shown that Imp-L2 binds human insulin specifically (Sloth Andersen et al. 2000), and that Imp-L2 inhibits the insulin signaling pathway (Honegger et al. 2006), we would have expected that the stimulation by human insulin would be less efficient in the presence of Imp-L2 in the medium. Because this was not the case, we wanted to test if the inhibition of Imp-L2 on human insulin stimulation was not observed due to the low binding affinity to human insulin. Therefore, we next tested if Imp-L2 interferes with Dilp2 induced stimulation of the insulin pathway. We were not able to stimulate stable Cl8-1 (containing MT-*IMPL2-HA*) cells with 10nM Dilp2 in the presence of Imp-L2, even though this concentration of Dilp2 nicely stimulated normal clon8 cells (see pPKB and pS6K phosphorylation in Fig. S30A+B). However, not only Cl8-1 cells in which Imp-L2 expression was induced but also the non-induced Cl8-1 cells displayed insensitivity to Dilp2 stimulation (lane 2 in Fig. S30A+B),

which was probably due to the leakiness of the MT-*Imp-L2-HA* construct. In these experiments $CuSO_4$ was added together with fresh media to the cells (lanes 1, 3, and 6). The medium of the control cells was exchanged at the same time (lanes 2, 4, and 6). Thus, the leaky MT-Imp-L2-HA could express and secrete protein for 14-15h. In an attempt to minimize the effect of the leaky *Imp-L2* expression we decreased the time of induction. By inducing Imp-L2 expression for only 6h we could indeed reduce the levels of extracellular Imp-L2 protein beyond detection limits in non-induced control cells (Fig. S30C). However, under these condition we could no longer detect the inhibitory effect of Imp-L2 on Dilp2 stimulation. Therefore, instead of lowering Imp-L2 concentration in the medium we next decided to increase the Dilp2 concentration used for stimulation. Indeed, we could only stimulate Cl8-1 cells in which Imp-L2 was not induced (Fig. S30D). Strangely, we only observed a difference between the phosphorylation of S6K and not PKB (see lanes 1+2 in Fig. S30D). Since we were not able to reproduce this result (Fig. S30E), no clear conclusion could be drawn if Imp-L2 hinders Dilp2 stimulation in cell culture.

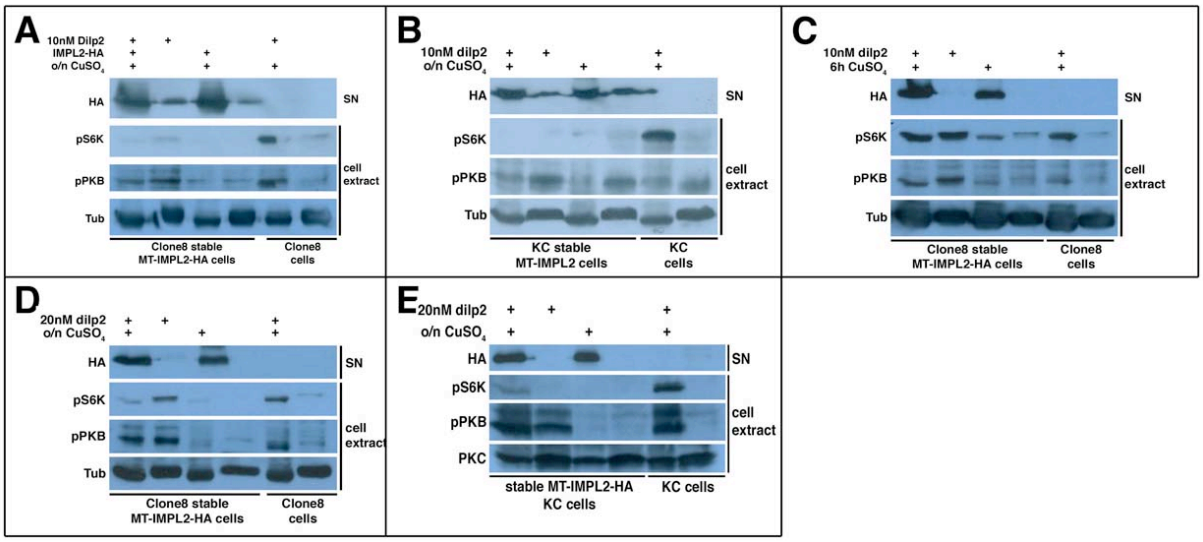


Figure S30: Stimulation of stable MT-*IMPL2-HA* cell lines with dilp2.

(A-E) For the cell extracts, 100µg of total protein extract was loaded, of the supernatants (SN) 60µl were loaded on a different gel. (A, B, D, E) Cells were starved in serum- and insulin-free medium (Shields & Sang supplemented with Penicillin/Streptomycin only) overnight. During this time $CuSO_4$ was added to the samples in lanes 1, 3, and 5. The cells were then stimulated with the indicated concentrations of dilp2 for 30min. The cells in (C) were starved and induced for only 6h, but otherwise treated the same way. The different sizes of the tubulin band are due to the presence of the $CuSO_4$, which was sometimes not washed away totally. "pS6K" refers to an Ab against phosphorylated dS6K, "pPKB" to an Ab against phosphorylated dPKB, "PKC" to an Ab against total PKCα.

Next, we wanted to test if addition of Imp-L2-containing supernatant decreases the sensitivity of wild-type cells to Dilp2 stimulation. Therefore, we collected supernatant of *Imp-L2* expressing cells and added different amounts of it to wild-type cells 30min prior to Dilp2 stimulation. Neither in starved (Fig. S31A) nor in non-starved (Fig. S31B) cells did the presence of Imp-L2 in the medium influence S6K or PKB phosphorylation. Since it is possible that Imp-L2 is diluted too much in the supernatants, we concentrated Imp-L2-containing supernatant by centrifugation using a Vivaspin6 concentrator (MWCO 10'000). But also with these concentrated Imp-L2-containing supernatants, we were not able to detect a reproducible effect on Dilp2 stimulation of wild-type cells (Fig. S31C+D).
Although we performed various different cell culture experiments, no unambiguous conclusions could be drawn if the inhibition of Dilp2 activity we saw in genetic experiments can be reproduced or studied in cell culture.

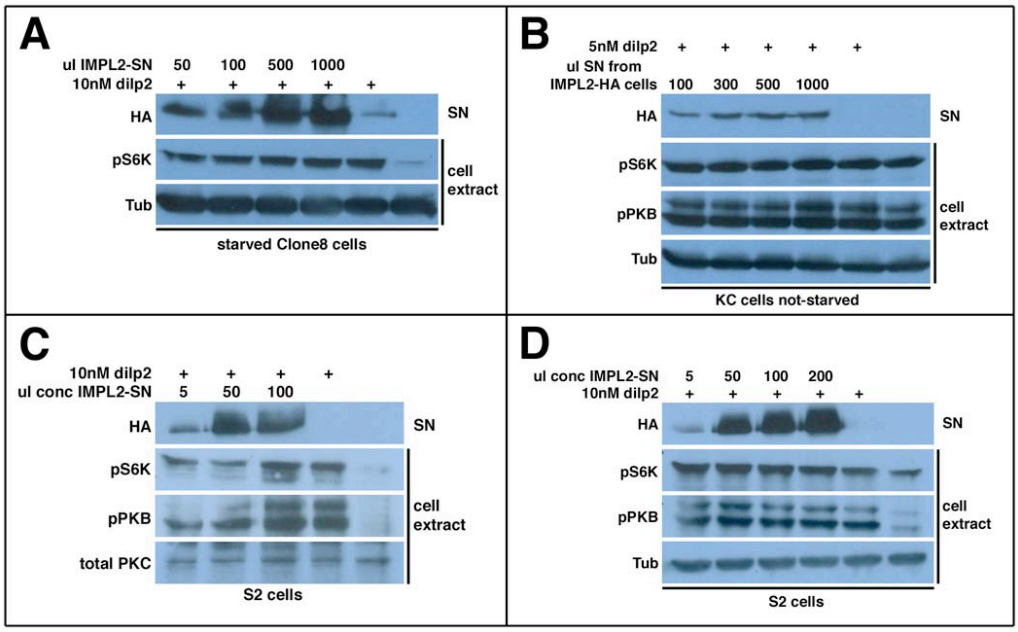


Figure S31: Addition of Imp-L2 containing medium does not affect insulin stimulation of cells.
(A+B) The supernatant of MT-IMPL2-HA containing Cl8-1 cells, induced o/n in Shields & Sang supplemented with Penicillin/Streptomycin, was added to o/n starved (A) or non-starved cells (B). After one hour, the cells were stimulated with indicated concentrations of recombinant dilp2 (P. Belawat). In (C+D) the supernatant of the Cl8-1 cells (collected as described above) was concentrated using a Vivaspin 6 concentrator (MWCO 10'000) before adding it to the cells. The following antibodies were used: mouse-anti-HA, rabbit-anti-phospho-S6K, rabbit-anti-phospho-PKB, rabbit-anti-total PKC, mouse-anti-tubulin.

m-NSCs specific expression of *shi*^{ts} only mildly affects body size

Recently it has been shown that ablation of the m-NSCs, which produce four of the seven dilps, is sufficient to decrease total body size (Ikeya et al. 2002; Rulifson et al. 2002). Using a temperature-sensitive dominant negative form of dynamin (UAS-*shi*^{ts}; (Kitamoto 2001) to reversibly block synaptic vesicle recycling and hence synaptic transmission, we wanted to assess if disturbed vesicle cycling in m-NSCs is sufficient to copy this size decrease due to inefficient or inexistent insulin secretion. Since this system is fast-responding and controlled by temperature it would provide the possibility to block insulin secretion at any time of development. We expressed UAS-*shi*^{ts} specifically in the m-NSCs by either the dilp2-55-*Gal4* or the dilp2-3-*Gal4* drivers and reared the flies either at the restrictive (29°C, red bars in Fig. S32) or the permissive temperature (22°C, blue bars in Fig. S32). As controls we expressed GFP and reaper in the m-NSCs. Disturbing vesicle cycling in the m-NSCs by *shi*^{ts} only slightly affected final body size (compare the red bars either with the blue ones or the GFP control in UAS-*shi*^{ts}, dilp2-3-*Gal4* flies, Fig. S32). Thus, secretion of insulin is presumably only marginally disturbed by *shi*^{ts} expression.

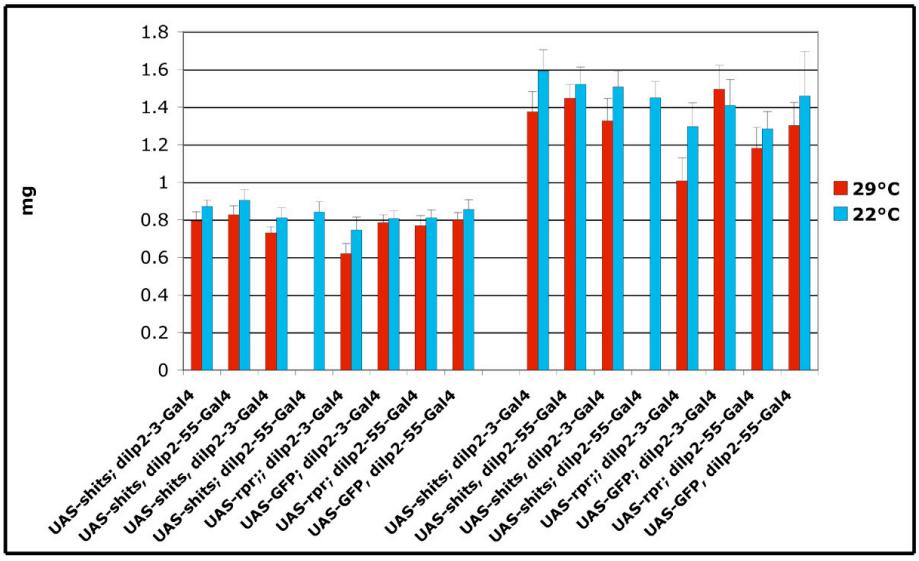


Figure S32: Blocking the synaptic transport of *dilp*-expressing m-NSC by expressing *shi*^{ts} is not sufficient to reproduce the m-NSC cell ablation phenotype.

Total body weights of flies expressing UAS-*shi*^{ts} in the *dilp*-producing m-NSCs. For this purpose the flies were reared in separate incubators at either 22°C (blue bars) or 29°C (red bars). Flies lacking the m-NSCs served as a positive control (dilp2-*Gal4*, UAS-*rpr*). The weights for the UAS-*shi*^{ts}, dilp2-55-*Gal4* cross at 29°C are missing, because these females died prior to egg deposition on 29°C.

Discussion

The evolutionary conserved insulin/IGF receptor pathway is involved in the control of a diverse array of processes such as growth, metabolism, longevity, and reproduction (Oldham and Hafen 2003). While insulin is a principal regulator of glucose and lipid metabolism (Saltiel and Kahn 2001), IGFs, as major downstream targets of growth hormone, are essential for regulating growth and body size (Nakae et al. 2001). Alterations in either insulin or IGF signaling are linked to severe disorders such as diabetes, obesity, polycystic ovarian disease, hyperlipidemia, hypertension, atherosclerosis, and cancer (Saltiel and Kahn 2001). Mutations in the tumor suppressor PTEN for instance, constitutively activates downstream components of this pathway such as PKB, leading to overproliferation and enhanced survival of mutant cells (Cantley and Neel 1999). Thus, PTEN deletion occurs in multiple tumor types, most prominently advanced glial tumors (glioblastoma multiforme/anaplastic astrocytoma), but also prostate, endometrial, renal and small cell lung carcinoma, melanoma, and meningioma (Li et al. 1997; Steck et al. 1997). The only other known negative regulator in the insulin signaling cascade is the TSC1/2 complex, which links the canonical insulin pathway with the TOR signaling network. Like PTEN, also the TSC1/2 complex acts as a tumor suppressor. The genes TSC1 and TSC2 are mutated in the severe human syndrome called Tuberous Sclerosis, which is characteristically associated with large benign tumors composed of large cells in the brain of patients (Pan et al. 2004). Because deregulation of the insulin signaling cascade is altering cellular growth, and thereby predisposing cells to become tumorigenic, the negative regulators of the pathway are all likely to function as tumor suppressors.

In order to identify novel inhibitors of the insulin signaling pathway, we conducted a gain-of-function (EP) screen for genes that suppress the InR induced hyperplasia in the *Drosophila* eye. Such an EP screen provides a complementary screening strategy to the general loss-of-function screens, in which genes lacking any obvious phenotype under standard culture conditions are likely to be missed. It is assumed that only one-third of all genes in the *Drosophila* genome can be mutated to an easily scored phenotype (Miklos and Rubin 1996). Since genes lacking a visible loss-of-function phenotype often cause a phenotype upon overexpression, an EP screen is a useful method to identify novel regulators of a pathway.

This PhD thesis describes the functional characterization of *Imp-L2*, which was found in such an EP screen based on its ability to suppress the *InR* induced big eye phenotype upon overexpression.

Imp-L2

The *Drosophila Imp-L2* gene was previously identified as a 20-hydroxyecdysone-induced gene encoding a secreted member of the immunoglobin superfamily (Garbe et al. 1993). We have shown that *Imp-L2* controls cell size as well as cell number non-autonomously by inhibiting insulin signaling at the level of the ligands. Although *Imp-L2* is not essential for survival under standard culture conditions, it is upregulated and necessary in larvae upon starvation to sustain extended periods of low nutrition.

The *Imp-L2* gene consists of three different transcripts (*RA*, *RB*, and *RC*) differing in the usage of the first exon, but all comprise the same protein coding region. While *Imp-L2-RA* and *Imp-L2-RC* have small non-coding first exons, *Imp-L2-RB* contains three small upstream open reading frames (uORFs) in its first exon. We have shown that these uORFs have an inhibitory effect on translation, since *Imp-L2-RB* constructs either lacking the first exon or containing mutated ATGs in the uORFs, all have stronger effects on growth than the full-length and wild-type transcript, when overexpressed. Also overexpression of *Imp-L2-RA* and *Imp-L2-RC* transcripts displayed stronger growth inhibition than *Imp-L2-RB*. Since all the UAS constructs of the different *Imp-L2* variants randomly integrated into the genome, their expression is differentially influenced by their surrounding. Therefore, to verify that the milder effect of UAS-*Imp-L2-RB* is really due to less efficient translation and not transcription, RNA levels must be measured by transcript-specific quantitative real-time PCR (qRT-PCR). Another alternative would be to generate new transgenic fly lines with the attB/attP system, which allows targeted integration of constructs at a specific landing site. Like this, the influence of the genomic region on transcription is the same for all the constructs. Another interesting question that could be addressed, once such transgenic fly lines with identical integration places of the UAS constructs is established, is, if like in the case of *GCN4*, whose translation rate is increased upon starvation (Hinnebusch 1997), the inhibiting effect of the uORFs on the translation changes under various stress conditions. Considering the upregulation of Imp-L2 protein expression under starvation and hypoxic

conditions, it is tempting to speculate that the *Imp-L2-RB* mRNA is transcribed constitutively, but only efficiently translated under specific stress conditions. The embryonic expression pattern supports such a hypothesis of a translational control mechanism, since mRNA can be detected at specific places throughout embryonic development, but Imp-L2 protein is only detected late in embryogenesis (Garbe et al. 1993). In such a case, the expression of *Imp-L2-RA* and *Imp-L2-RC* need to be under more restrictive control at the level of transcription, since their translation without uORFs would work efficiently. To proof this, the abundance of the different transcripts during development and under various conditions should be assayed. Another possibility would be to generate reporter constructs of the different transcripts, luciferase for cell culture and GFP or lacZ for an *in vivo* analysis, which would also allow monitoring the level of transcript expression under diverse conditions.

Apart from its function as a regulator of insulin signaling upon unfavorable environmental conditions, Imp-L2 might also play a role during development by attenuating insulin signaling. It has been shown that ecdysone, besides its role as a hormone controlling developmental timing and the onset of metamorphosis, negatively regulates insulin signaling (Rusten et al. 2004; Colombani et al. 2005). However, it is unknown, what the connection between ecdysone- and insulin signaling is. Therefore, as an ecdysone inducible protein that efficiently inhibits Dilp2 signaling, Imp-L2 is a good candidate for being the link between the two pathways. Flies overexpressing *PI3K* in the ring gland (P0206>*dp110*) display slightly increased ecdysone levels, which is sufficient to downregulate insulin signaling in the fat body and to decrease total body size (Colombani et al. 2005). Since this phenotype was not reproducible on our fly food, it could not be tested if the size decrease of P02026>*dp110* flies persists in an *Imp-L2* null background. Additionally, the Léopold Group could not detect any change in *Imp-L2* mRNA levels in larvae with such a mildly upregulated ecdysone levels (tested by RT-PCR, personal communication P.Léopold, Nice). However, it is still possible that ecdysone interacts not only on the transcriptional level with *Imp-L2*, but affects its translation or secretion. Recently, it has been shown that insulin and ecdysone signaling are also connected in controlling autophagy. Genetic interaction studies showed that ecdysone signaling autonomously downregulates PI3K signaling, which represents the effector mechanism for induction of programmed autophagy (Rusten et al. 2004). Since the connection between ecdysone and insulin signaling is strictly cell-autonomous it is rather unlikely that Imp-L2 relays the ecdysone signal in this context.

Comparison of the vertebrate and invertebrate insulin binding proteins

Although in insects and other lower organisms, as for example nematodes, the insulin signaling pathway and insulin-like proteins are highly conserved, clear orthologs of IGFBPs are missing. In this thesis we describe Imp-L2, the first protein in insects not only possessing the ability to bind and regulate insulin *in vivo*, but is in its C-terminus also shows homology to IGFBP-rP1, the only insulin binding protein in vertebrates known to bind strongly to insulin. Further, Imp-L2, like the putative tumor suppressor IGFBP-rP1, always showed an inhibitory effect on growth and interacts with Dilp2, the closest homolog of human insulin in *Drosophila*. Thus, Imp-L2 acts as a functional homolog of IGFBPs in insects. An analysis of IGFBP-rP1 expression in 60 primary breast cancers using immunohistochemistry revealed that 12 normal and benign breast tissues had strong IGFBP-rP1 expression, 16 ductal carcinomas *in situ* showed weak IGFBP-rP1 levels and the invasive carcinomas were negative for IGFBP-rP1, all consistent with its role as a tumor suppressor (Burger et al. 1998). Through its role in controlling insulin activity under unfavorable conditions like starvation and hypoxia, Imp-L2 also has the potential to be a tumor suppressor, and since the conditions inside a tumor are often highly unfavorable for a cell, Imp-L2 activity would have to be abolished to sustain growth. However, expression of IGFBP-rP1 in flies was not able to mimic the effects of Imp-L2 overexpression. It is possible that the N-terminus of IGFBP-rP1, which is highly homologous to the evolutionary non-conserved IGFBPs, disturbs the interaction with *Drosophila* insulins. Thus, it might be possible to mimic the effects of Imp-L2 by a chimeric protein consisting of the Imp-L2 N- and the IGFBP-rP1 C-terminus.
The polycystic ovarian syndrom (PCOS), a mammalian disorder, in the course of which IGFBPs are assumed to play an important role, is reminiscent of the big ovary phenotype of *Imp-L2* mutant females. PCOS is a disorder of unknown, probably heterogeneous etiology, characterized by chronic anovulation, hyperandrogenism, and enlarged polycystic ovaries (for a review see (Poretsky et al. 1999)). PCOS affects between 5-10% of women at reproductive state. Although the phenotype of the enlarged ovaries of *Imp-L2* mutant females resembles the large polycystic ovaries of humans, the cause for these polycystic ovaries is different in both situations. While anovulation is a major cause in humans, *Imp-L2* mutant females display no failure in egg deposition, but rather show enhanced egg production. Thus, the ovulation rate is unchanged in *Drosophila*. Even though the development of the different situations differs considerably, it is striking how similar the phenotypes are.

Albeit the classical *IGFBPs* are not present in the *Drosophila* genome, it contains an ortholog of *ALS*. It has been shown in mammals that in postnatal serum most IGFs are sequestered into ternary complexes consisting of one molecule each of IGF, IGFBP-3 or IGFBP-5, and ALS (Boisclair et al. 2001). Since insects lack any orthologs of IGFBP-3 and IGFBP-5, and an ALS-IGFBP-rP1 binding has never been shown in vertebrates, it is unclear what the binding partners of ALS are in *Drosophila*. However, like in mammals (Dai et al. 1994; Oster et al. 1996), *dALS* is expressed mainly by the fat body, the liver analog of insects, and its expression is sensitive to starvation (Colombani et al. 2003). It remains to be tested whether ALS for the lack of canonical IGFBPs in insects binds insulin directly, whether it binds to binary Imp-L2-insulin complexes, or whether it binds Imp-L2 alone. So far, we were not able to generate a clear *dALS* phenotype or interaction in conjunction with either *dilp2* or *Imp-L2*. In vitro pull-downs and further genetic interaction studies should enlighten the function of ALS in *Drosophila* and clarify its role in modulating insulin signaling.

Interaction of Imp-L2 with the Dilps

We have shown that besides its interaction with *dilp2* (Honegger et al. 2006), *Imp-L2* interacts at least genetically also with *dilp5* and *dilp6*. In conjunction with the other dilps we were not able to detect a genetic interaction. This was partly due to the lack of a reproducible overexpression phenotype of *dilp1*, *3*, *4*, and *7*. Their expression was also not sufficient to additionally increase the size of *Imp-L2* mutants. Thus, we can only conclude, that Imp-L2 interacts with *dilp2*, *5*, and *6*, albeit a putative interaction with the other *dilps* cannot be excluded.

Since there exist no mutants for the different *dilps*, it is crucial to test if really all the dilps are capable of influencing total body size, as stated before (Ikeya et al. 2002). Although we were not able to reproduce this result, the overexpression of *dilp1*, *3*, *4*, and *7* could be repeated in a sensitized system. Direct constructs overexpressing the *InR* specifically in the eye under the control of the GMR promotor (GMR-InR^{wt}), display weak or no phenotypes. Therefore, overexpressing *dilp1*, *3*, *4*, or *7* by GMR-*Gal4* in a GMR-InR^{wt} background rather shows an effect on eye size than in wild-type situations. Further, this experiment may provide a hint, if all the dilps signal through the InR. A possible phenotype of one of the *dilps* could then be further tested in an *Imp-L2* loss-of-function background to test a possible interaction.

Additionally, co-overexpression of *Imp-L2* and either *dilp1*, *3*, *4*, or *7* might hint at a putative interaction.
Another way of showing a potential interaction of Imp-L2 with the dilps would be to tag Dilp1, and 3-7 N-terminally in the same way as Dilp2. In an *in vitro* pull-down assay the physical interaction of Imp-L2 with the different Dilps could then be monitored.
Obviously, the best environment to study the Imp-L2-Dilp interaction would be directly in the hemolymph. Since both proteins are secreted and show non-autonomous effects it is most likely that their interaction takes place in this fluid. The limiting parameters of such an experiment would be on one hand the scarce amount of hemolymph which can be extracted from a single fly/larva, and on the other hand the dilp antibodies, which are presumably not good enough for a pull-down experiment. Thus, tagged proteins could be used to perform an immunopurification from pooled hemolymph samples of about 100 larvae. Since immunohistochemistry is presumably not sensitive enough to see a potential interaction in the hemolymph, quantitative proteomics could be used to detect the proteins in the sample. If the conditions to conduct such an experiment could be established, this assay could be also used to determine the interaction of the dilps with Imp-L2 at different developmental stages and under various stress conditions.

Nutrient-dependent regulation of growth by Imp-L2

As we have already described in the introduction, growth of an organism is controlled by the combination of genetic and environmental means. The basic growth information is stored as genetic information that constantly adapts according to environmental changes. Although there has lately been made a great amount of progress in understanding both the genetic pathways and the environmental cues controlling growth, it has so far remained obscure, how an organism orchestrates the different signals to generate a common growth output. It is crucial for an animal to be capable of reacting immediately to a changing environment and attenuate the genetic programs according to those changes. One of the central pathways in the control of metabolism and growth is the IIS cascade. Therefore, IIS is one of the major targets which needs to be adjusted following such changes in environmental conditions. One way to achieve this, is to change the transcription of the various pathway members. However, transcriptional alterations change the activity of the pathway only slowly. Another way is to modulate the activity of the pathway directly at the protein level. In vertebrates, the IGFBP

superfamily functions that way. By directly binding to the IGFs and/or insulin, these proteins possess the ability to regulate the availability, and therefore also the activity of the IIS pathway at an extracellular level. Consequently, this provides a fast response to the changing environmental conditions.

In this thesis we provide evidence that Imp-L2 acts as a *Drosophila* equivalent to the vertebrate IGFBPs. Imp-L2 is the first insulin binding protein identified in invertebrates. It acts as negative regulator of IIS in *Drosophila* by controlling the activity of the most potent growth-inducing insulin, Dilp2. Although *Imp-L2* is not essential for survival of the flies under standard culture conditions, it is necessary to restrict the activity of the IIS pathway when nutrients become limiting. It is of uttermost importance for an organism to respond rapidly to starvation conditions by adapting metabolism and growth, because unconfined growth under scarce nutritional conditions swiftly results in lethality (Britton et al. 2002). While the transcription of *dilp3* and *5* is downregulated upon starvation conditions, *dilp2* mRNA levels are unaffected by nutritional availability. Imp-L2 binding provides the only known mechanism so far, that allows the restriction of Dilp2 activity upon unfavorable environmental conditions. Thus, the necessity of *Imp-L2* under starvation conditions presumably emerges from its inhibitory effect on Dilp2 activity. Like this, Imp-L2 acts as a link between environmental conditions such as starvation and the IIS cascade to control growth and metabolism in *Drosophila*.

One of the major challenges faced by all organisms during evolution was to ensure survival of periods in which nutrient availability is low. It is therefore not surprising that mechanisms evolved early in evolution to meet this challenge. The genetic and biochemical studies presented in this thesis highlight the high degree of functional conservation between Imp-L2 and the IGFBP system used in higher organisms to shuttle and control insulin and IGFs. They support the hypothesis that these molecules represent an essential part of the cellular and organismal mechanisms that allow the attenuation of cellular and organismal growth in response to nutrients.

Materials and Methods

Material and Methods used in the course of this study are essentially described in the manuscript (Honegger et al. 2006)

References

Accili, D., Kido, Y., Nakae, J., Lauro, D., and Park, B.C. 2001. Genetics of type 2 diabetes: insight from targeted mouse mutants. *Curr Mol Med* **1**(1): 9-23.

Arquier, N., Bourouis, M., Colombani, J., and Leopold, P. 2005. Drosophila Lk6 kinase controls phosphorylation of eukaryotic translation initiation factor 4E and promotes normal growth and development. *Curr Biol* **15**(1): 19-23.

Azevedo, R.B., French, V., and Partridge, L. 2002. Temperature modulates epidermal cell size in Drosophila melanogaster. *J Insect Physiol* **48**(2): 231-237.

Baxter, R.C. and Martin, J.L. 1989. Structure of the Mr 140,000 growth hormone-dependent insulin-like growth factor binding protein complex: determination by reconstitution and affinity-labeling. *Proc Natl Acad Sci U S A* **86**(18): 6898-6902.

Bernal, A. and Kimbrell, D.A. 2000. Drosophila Thor participates in host immune defense and connects a translational regulator with innate immunity. *Proc Natl Acad Sci U S A* **97**(11): 6019-6024.

Bjornsti, M.A. and Houghton, P.J. 2004. The TOR pathway: a target for cancer therapy. *Nat Rev Cancer* **4**(5): 335-348.

Bohni, R., Riesgo-Escovar, J., Oldham, S., Brogiolo, W., Stocker, H., Andruss, B.F., Beckingham, K., and Hafen, E. 1999. Autonomous control of cell and organ size by CHICO, a Drosophila homolog of vertebrate IRS1-4. *Cell* **97**(7): 865-875.

Boisclair, Y.R., Rhoads, R.P., Ueki, I., Wang, J., and Ooi, G.T. 2001. The acid-labile subunit (ALS) of the 150 kDa IGF-binding protein complex: an important but forgotten component of the circulating IGF system. *J Endocrinol* **170**(1): 63-70.

Britton, J.S., Lockwood, W.K., Li, L., Cohen, S.M., and Edgar, B.A. 2002. Drosophila's insulin/PI3-kinase pathway coordinates cellular metabolism with nutritional conditions. *Developmental Cell* **2**(2): 239-249.

Brogiolo, W., Stocker, H., Ikeya, T., Rintelen, F., Fernandez, R., and Hafen, E. 2001. An evolutionarily conserved function of the Drosophila insulin receptor and insulin-like peptides in growth control. *Curr Biol* **11**(4): 213-221.

Brugarolas, J., Lei, K., Hurley, R.L., Manning, B.D., Reiling, J.H., Hafen, E., Witters, L.A., Ellisen, L.W., and Kaelin, W.G., Jr. 2004. Regulation of mTOR function in response

to hypoxia by REDD1 and the TSC1/TSC2 tumor suppressor complex. *Genes Dev* **18**(23): 2893-2904.

Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J., and Greenberg, M.E. 1999. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* **96**(6): 857-868.

Burger, A.M., Leyland-Jones, B., Banerjee, K., Spyropoulos, D.D., and Seth, A.K. 2005. Essential roles of IGFBP-3 and IGFBP-rP1 in breast cancer. *Eur J Cancer* **41**(11): 1515-1527.

Burger, A.M., Zhang, X., Li, H., Ostrowski, J.L., Beatty, B., Venanzoni, M., Papas, T., and Seth, A. 1998. Down-regulation of T1A12/mac25, a novel insulin-like growth factor binding protein related gene, is associated with disease progression in breast carcinomas. *Oncogene* **16**(19): 2459-2467.

Cantley, L.C. and Neel, B.G. 1999. New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. *Proc Natl Acad Sci U S A* **96**(8): 4240-4245.

Cao, C. and Brown, M.R. 2001. Localization of an insulin-like peptide in brains of two flies. *Cell & Tissue Research* **304**(2): 317-321.

Casey, O.M., Fitzpatrick, R., McInerney, J.O., Morris, D.G., Powell, R., and Sreenan, J.M. 2004. Analysis of gene expression in the bovine corpus luteum through generation and characterisation of 960 ESTs. *Biochim Biophys Acta* **1679**(1): 10-17.

Chassin, D., Laurent, A., Janneau, J.L., Berger, R., and Bellet, D. 1995. Cloning of a new member of the insulin gene superfamily (INSL4) expressed in human placenta. *Genomics* **29**(2): 465-470.

Cheadle, J.P., Reeve, M.P., Sampson, J.R., and Kwiatkowski, D.J. 2000. Molecular genetic advances in tuberous sclerosis. *Hum Genet* **107**(2): 97-114.

Chen, C., Jack, J., and Garofalo, R.S. 1996. The Drosophila insulin receptor is required for normal growth. *Endocrinology* **137**(3): 846-856.

Clancy, D.J., Gems, D., Harshman, L.G., Oldham, S., Stocker, H., Hafen, E., Leevers, S.J., and Partridge, L. 2001. Extension of life-span by loss of CHICO, a Drosophila insulin receptor substrate protein. *Science* **292**(5514): 104-106.

Clemmons, D.R. 1999. Insulin-like growth factor binding proteins. in *Handbook of Physiology, Section 7: The Endocrine System, Volume V: Hormonal Control of Growth* (ed. H.M. Goodman), pp. 573-633. Oxford University Press, New York.

Colombani, J., Bianchini, L., Layalle, S., Pondeville, E., Dauphin-Villemant, C., Antoniewski, C., Carre, C., Noselli, S., and Leopold, P. 2005. Antagonistic actions of ecdysone and insulins determine final size in Drosophila. *Science* **310**(5748): 667-670.

Colombani, J., Raisin, S., Pantalacci, S., Radimerski, T., Montagne, J., and Leopold, P. 2003. A nutrient sensor mechanism controls Drosophila growth. *Cell* **114**(6): 739-749.

Conklin, D., Lofton-Day, C.E., Haldeman, B.A., Ching, A., Whitmore, T.E., Lok, S., and Jaspers, S. 1999. Identification of INSL5, a new member of the insulin superfamily. *Genomics* **60**(1): 50-56.

Conlon, I. and Raff, M. 1999. Size control in animal development. *Cell* **96**(2): 235-244.

Currie, R.A., Walker, K.S., Gray, A., Deak, M., Casamayor, A., Downes, C.P., Cohen, P., Alessi, D.R., and Lucocq, J. 1999. Role of phosphatidylinositol 3,4,5-trisphosphate in regulating the activity and localization of 3-phosphoinositide-dependent protein kinase-1. *Biochem J* **337 (Pt 3)**: 575-583.

Dai, J., Scott, C.D., and Baxter, R.C. 1994. Regulation of the acid-labile subunit of the insulin-like growth factor complex in cultured rat hepatocytes. *Endocrinology* **135**(3): 1066-1072.

Dai, J.D. and Gilbert, L.I. 1991. Metamorphosis of the corpus allatum and degeneration of the prothoracic glands during the larval-pupal-adult transformation of Drosophila melanogaster: a cytophysiological analysis of the ring gland. *Dev Biol* **144**(2): 309-326.

Dietz, A. 2003. A phenotypic characterization of a temperature-sensitive PI3K allele in *Drosophila melanogaster*. In *Zoologisches Institut*, pp. 101. Universität Zürich, Zürich.

Domene, H.M., Bengolea, S.V., Jasper, H.G., and Boisclair, Y.R. 2005. Acid-labile subunit deficiency: phenotypic similarities and differences between human and mouse. *J Endocrinol Invest* **28**(5 Suppl): 43-46.

Dominguez, F., Avila, S., Cervero, A., Martin, J., Pellicer, A., Castrillo, J.L., and Simon, C. 2003. A combined approach for gene discovery identifies insulin-like growth factor-binding protein-related protein 1 as a new gene implicated in human endometrial receptivity. *J Clin Endocrinol Metab* **88**(4): 1849-1857.

Dong, J. and Pan, D. 2004. Tsc2 is not a critical target of Akt during normal Drosophila development. *Genes Dev* **18**(20): 2479-2484.

Drummond-Barbosa, D. and Spradling, A.C. 2001. Stem cells and their progeny respond to nutritional changes during Drosophila oogenesis. *Dev Biol* **231**(1): 265-278.

Dun, S.L., Brailoiu, E., Wang, Y., Brailoiu, G.C., Liu-Chen, L.Y., Yang, J., Kang Chang, J., and Dun, N.J. 2006. Insulin-like peptide 5: expression in the mouse brain and mobilization of calcium*. *Endocrinology*.

Duve, H., Thorpe, A., and Lazarus, N.R. 1979. Isolation of material displaying insulin-like immunological biological activity from the brain of the blowfly Calliphora vomitoria. *Biochem J* **184**(2): 221-227.

Edgar, B.A. and Orr-Weaver, T.L. 2001. Endoreplication cell cycles: more for less. *Cell* **105**(3): 297-306.

Efstratiadis, A. 1998. Genetics of mouse growth. *Int J Dev Biol* **42**(7): 955-976.

Fernandez, R., Tabarini, D., Azpiazu, N., Frasch, M., and Schlessinger, J. 1995. The Drosophila insulin receptor homolog: a gene essential for embryonic development encodes two receptor isoforms with different signaling potential. *Embo J* **14**(14): 3373-3384.

Fernandez-Almonacid, R. and Rosen, O.M. 1987. Structure and ligand specificity of the Drosophila melanogaster insulin receptor. *Mol Cell Biol* **7**(8): 2718-2727.

French, V., Feast, M., and Partridge, L. 1998. Body size and cell size in Drosophila: the developmental response to temperature. *J Insect Physiol* **44**(11): 1081-1089.

Froguel, P., Velho, G., Passa, P., and Cohen, D. 1993. Genetic determinants of type 2 diabetes mellitus: lessons learned from family studies. *Diabete Metab* **19**(1): 1-10.

Galic, M. 2001. The antagonistic role of *Drosophila* Insulin Like Peptides (DILPs) and Imaginal Morphogenesis Protein - Late 2 (IMP-L2) in the *Drosophila* insulin receptor signaling pathway

. In *Zoologisches Institut*. Universität Zürich, Zürich.

Gao, X., Neufeld, T.P., and Pan, D. 2000. Drosophila PTEN regulates cell growth and proliferation through PI3K-dependent and -independent pathways. *Dev Biol* **221**(2): 404-418.

Gao, X. and Pan, D. 2001. TSC1 and TSC2 tumor suppressors antagonize insulin signaling in cell growth. *Genes Dev* **15**(11): 1383-1392.

Garami, A., Zwartkruis, F.J., Nobukuni, T., Joaquin, M., Roccio, M., Stocker, H., Kozma, S.C., Hafen, E., Bos, J.L., and Thomas, G. 2003. Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2. *Mol Cell* **11**(6): 1457-1466.

Garbe, J.C., Yang, E., and Fristrom, J.W. 1993. IMP-L2: an essential secreted immunoglobulin family member implicated in neural and ectodermal development in Drosophila. *Development* **119**(4): 1237-1250.

Garofalo, R.S. 2002. Genetic analysis of insulin signaling in Drosophila. *Trends Endocrinol Metab* **13**(4): 156-162.

Gavin, J., Alberti, K., Davidson, M., DeFronzo, R., Drash, A., Gabbe, S., Genuth, S., Harris, M., Kahn, R., Keen, H., Knowler, W., Lebovitz, H., Maclaren, N., Palmer, J., Raskin, P., Rizza, R., and Stern, M. 2000. Report of the Expert Committee on the diagnosis and classification of diabetes mellitus. *Diabetes Care* **23**: S4-S19.

Goberdhan, D.C., Paricio, N., Goodman, E.C., Mlodzik, M., and Wilson, C. 1999. Drosophila tumor suppressor PTEN controls cell size and number by antagonizing the Chico/PI3-kinase signaling pathway. *Genes Dev* **13**(24): 3244-3258.

Goldsworthy, G.J. 1970. The action of hyperglycaemic factors from the corpus cardiacum of Locusta migratoria on glycogen phosphorylase. *Gen Comp Endocrinol* **14**(1): 78-85.

Green, A., Christian Hirsch, N., and Pramming, S.K. 2003. The changing world demography of type 2 diabetes. *Diabetes Metab Res Rev* **19**(1): 3-7.

Groth, A.C., Fish, M., Nusse, R., and Calos, M.P. 2004. Construction of transgenic Drosophila by using the site-specific integrase from phage phiC31. *Genetics* **166**(4): 1775-1782.

Guertin, D.A., Kim, D.H., and Sabatini, D.M. 2004. Growth control through the mTOR network. in *Cell Growth: Control of Cell Size* (ed. M.N. Hall, M. Raff, and G. Thomas), pp. 193-234. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Guler, H.P., Zapf, J., Schmid, C., and Froesch, E.R. 1989. Insulin-like growth factors I and II in healthy man. Estimations of half-lives and production rates. *Acta Endocrinol (Copenh)* **121**(6): 753-758.

Gäde, G. 1981. Activation of fat body glycogen phosphorylase in *Locusta migratoria* by corpus cardiacum extracts and synthetic adipokinetic hormone. *J Insect Physiol* **27**: 155-161.

Gäde, G., Wilps, H., and Kellner, R. 1990. Isolation and structure of a novel charged member of the red-pigment-concentrating hormone-adipokinetic hormone family of peptides isolated from the corpora cardiaca of the blowfly Phormia terraenovae (Diptera). *Biochem J* **269**(2): 309-313.

Hafen, E. 2004. Cancer, type 2 diabetes, and ageing: news from flies and worms. *Swiss Med Wkly* **134**(49-50): 711-719.

Hanada, M., Feng, J., and Hemmings, B.A. 2004. Structure, regulation and function of PKB/AKT--a major therapeutic target. *Biochim Biophys Acta* **1697**(1-2): 3-16.

Hanahan, D. and Weinberg, R.A. 2000. The hallmarks of cancer. *Cell* **100**(1): 57-70.

Harvie, P.D., Filippova, M., and Bryant, P.J. 1998. Genes expressed in the ring gland, the major endocrine organ of Drosophila melanogaster. *Genetics* **149**(1): 217-231.

Hasselbalch, S.G., Knudsen, G.M., Jakobsen, J., Hageman, L.P., Holm, S., and Paulson, O.B. 1995. Blood-brain barrier permeability of glucose and ketone bodies during short-term starvation in humans. *Am J Physiol* **268**(6 Pt 1): E1161-1166.

Heitman, J., Movva, N.R., and Hall, M.N. 1991. Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science* **253**(5022): 905-909.

Hill, M.M. and Hemmings, B.A. 2002. Inhibition of protein kinase B/Akt. implications for cancer therapy. *Pharmacol Ther* **93**(2-3): 243-251.

Hinnebusch, A.G. 1997. Translational regulation of yeast GCN4. A window on factors that control initiator-trna binding to the ribosome. *J Biol Chem* **272**(35): 21661-21664.

Honegger, B., Galic, M., Köhler, K., Brogiolo, W., and Hafen, E. 2006. Imp-L2, the first insulin binding protein identified in *Drosophila melanogaster*, binds Dilp2 and counteracts its activity. In *To be submitted*.

Hsu, S.Y., Nakabayashi, K., Nishi, S., Kumagai, J., Kudo, M., Sherwood, O.D., and Hsueh, A.J. 2002. Activation of orphan receptors by the hormone relaxin. *Science* **295**(5555): 671-674.

Huang, H., Potter, C.J., Tao, W., Li, D.M., Brogiolo, W., Hafen, E., Sun, H., and Xu, T. 1999. PTEN affects cell size, cell proliferation and apoptosis during Drosophila eye development. *Development* **126**(23): 5365-5372.

Hwa, V., Oh, Y., and Rosenfeld, R.G. 1999. The insulin-like growth factor-binding protein (IGFBP) superfamily. *Endocrine Reviews* **20**(6): 761-787.

Ikeya, T., Galic, M., Belawat, P., Nairz, K., and Hafen, E. 2002. Nutrient-dependent expression of insulin-like peptides from neuroendocrine cells in the CNS contributes to growth regulation in Drosophila. *Curr Biol* **12**(15): 1293-1300.

Inoki, K., Li, Y., Zhu, T., Wu, J., and Guan, K.L. 2002. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat Cell Biol* **4**(9): 648-657.

Inoki, K., Zhu, T., and Guan, K.L. 2003. TSC2 mediates cellular energy response to control cell growth and survival. *Cell* **115**(5): 577-590.

Jacinto, E., Loewith, R., Schmidt, A., Lin, S., Ruegg, M.A., Hall, A., and Hall, M.N. 2004. Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. *Nat Cell Biol* **6**(11): 1122-1128.

Johnston, G.C., Pringle, J.R., and Hartwell, L.H. 1977. Coordination of growth with cell division in the yeast Saccharomyces cerevisiae. *Exp Cell Res* **105**(1): 79-98.

Jones, J.I. and Clemmons, D.R. 1995. Insulin-like growth factors and their binding proteins: biological actions. *Endocr Rev* **16**(1): 3-34.

Junger, M.A., Rintelen, F., Stocker, H., Wasserman, J.D., Vegh, M., Radimerski, T., Greenberg, M.E., and Hafen, E. 2003. The Drosophila forkhead transcription factor FOXO mediates the reduction in cell number associated with reduced insulin signaling. *J Biol* **2**(3): 20.

Kandt, R.S. 2002. Tuberous sclerosis complex and neurofibromatosis type 1: the two most common neurocutaneous diseases. *Neurol Clin* **20**(4): 941-964.

Kato, M.V. 2000. A secreted tumor-suppressor, mac25, with activin-binding activity. *Mol Med* **6**(2): 126-135.

Kim, S.K. and Rulifson, E.J. 2004. Conserved mechanisms of glucose sensing and regulation by Drosophila corpora cardiaca cells. *Nature* **431**(7006): 316-320.

King, R.C., Aggarwal, S.K., and Bodenstein, D. 1966. The comparative submicroscopic morphology of the ring gland of Drosophila melanogaster during the second and third larval instars. *Z Zellforsch Mikrosk Anat* **73**(2): 272-285.

Kitamoto, T. 2001. Conditional modification of behavior in Drosophila by targeted expression of a temperature-sensitive shibire allele in defined neurons. *J Neurobiol* **47**(2): 81-92.

Kramer, J.M., Davidge, J.T., Lockyer, J.M., and Staveley, B.E. 2003. Expression of Drosophila FOXO regulates growth and can phenocopy starvation. *BMC Dev Biol* **3**: 5.

Lafont, R. and Horn, D.H.S. 1989. Phytoecdysteroids: Structure and occurence. in *Ecdysone: From Chemistry to Mode of Action* (ed. J. Koolman), pp. 39-64. Thieme-Verlag, Stauttgart.

Laurent, A., Rouillac, C., Delezoide, A.L., Giovangrandi, Y., Vekemans, M., Bellet, D., Abitbol, M., and Vidaud, M. 1998. Insulin-like 4 (INSL4) gene expression in human embryonic and trophoblastic tissues. *Mol Reprod Dev* **51**(2): 123-129.

Leevers, S.J. and Hafen, E. 2004. Growth Regulation by Insulin and TOR Signaling in *Drosophila.* in *Cell Growth: Control of Cell Size* (ed. M.N. Hall, M. Raff, and G.

Thomas), pp. 167-192. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Levine, B. and Klionsky, D.J. 2004. Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Dev Cell* **6**(4): 463-477.

Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S.I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., Bigner, S.H., Giovanella, B.C., Ittmann, M., Tycko, B., Hibshoosh, H., Wigler, M.H., and Parsons, R. 1997. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* **275**(5308): 1943-1947.

Li, Y., Corradetti, M.N., Inoki, K., and Guan, K.L. 2004. TSC2: filling the GAP in the mTOR signaling pathway. *Trends Biochem Sci* **29**(1): 32-38.

Liu, G., Roy, J., and Johnson, E.A. 2006. Identification and function of hypoxia-response genes in Drosophila melanogaster. *Physiol Genomics* **25**(1): 134-141.

Lizcano, J.M., Alrubaie, S., Kieloch, A., Deak, M., Leevers, S.J., and Alessi, D.R. 2003. Insulin-induced Drosophila S6 kinase activation requires phosphoinositide 3-kinase and protein kinase B. *Biochem J* **374**(Pt 2): 297-306.

Loewith, R. and Hall, M.N. 2004. TOR signaling on yeast: temporal and spatial control of cell growth. in *Cell Growth: Control of Cell Size* (ed. M.N. Hall, M. Raff, and G. Thomas), pp. 139-165. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Lok, S., Johnston, D.S., Conklin, D., Lofton-Day, C.E., Adams, R.L., Jelmberg, A.C., Whitmore, T.E., Schrader, S., Griswold, M.D., and Jaspers, S.R. 2000. Identification of INSL6, a new member of the insulin family that is expressed in the testis of the human and rat. *Biol Reprod* **62**(6): 1593-1599.

Long, X., Lin, Y., Ortiz-Vega, S., Yonezawa, K., and Avruch, J. 2005. Rheb binds and regulates the mTOR kinase. *Curr Biol* **15**(8): 702-713.

Louvi, A., Accili, D., and Efstratiadis, A. 1997. Growth-promoting interaction of IGF-II with the insulin receptor during mouse embryonic development. *Dev Biol* **189**(1): 33-48.

Lund, P.K. 1999. Insulin-like growth factors: gene structure and regulation. in *Handbook of Physiology, Section 7: The Endocrine System, Volume V: Hormonal Control of Growth* (ed. H.M. Goodman), pp. 537-573. Oxford University Press, New York.

Madhavan, M.M. and Schneiderman, H.A. 1977. Histological analysis of the dynamics of growth of imaginal discs and histoblast nests during the larval development of *Drosophila melanogaster*. *Wilhelm Roux' Arch Entwicklungs Mech Org* **183**: 269-305.

Maehama, T. and Dixon, J.E. 1998. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* **273**(22): 13375-13378.

Manning, B.D. 2004. Balancing Akt with S6K: implications for both metabolic diseases and tumorigenesis. *J Cell Biol* **167**(3): 399-403.

Martin, D.E. and Hall, M.N. 2005. The expanding TOR signaling network. *Curr Opin Cell Biol* **17**(2): 158-166.

Martin, J.L. and Baxter, R.C. 1986. Insulin-like growth factor-binding protein from human plasma. Purification and characterization. *J Biol Chem* **261**(19): 8754-8760.

McCall, A.L. 1993. Effects of glucose deprivation on glucose metabolism in the central nervous system. in *Hypoglycemia and Diabetes, Clinical and Physiological Aspects* (ed. B.M. Frier and B.M. Fisher), pp. 56-71. Edward Arnold, London.

Meijer, A.J. and Codogno, P. 2004. Regulation and role of autophagy in mammalian cells. *Int J Biochem Cell Biol* **36**(12): 2445-2462.

Miklos, G.L. and Rubin, G.M. 1996. The role of the genome project in determining gene function: insights from model organisms. *Cell* **86**(4): 521-529.

Montagne, J., Radimerski, T., and Thomas, G. 2001. Insulin signaling: lessons from the Drosophila tuberous sclerosis complex, a tumor suppressor. *Sci STKE* **2001**(105): PE36.

Montagne, J., Stewart, M.J., Stocker, H., Hafen, E., Kozma, S.C., and Thomas, G. 1999. Drosophila S6 kinase: a regulator of cell size. *Science* **285**(5436): 2126-2129.

Mordue, W. and Goldsworthy, G.J. 1969. The physiological effects of corpus cardiacum extracts in locusts. *Gen Comp Endocrinol* **12**(2): 360-369.

Murphy, L.J. 1998. Insulin-like growth factor-binding proteins: functional diversity or redundancy? *J Mol Endocrinol* **21**(2): 97-107.

Murphy, M., Pykett, M.J., Harnish, P., Zang, K.D., and George, D.L. 1993. Identification and characterization of genes differentially expressed in meningiomas. *Cell Growth Differ* **4**(9): 715-722.

Nakae, J., Kido, Y., and Accili, D. 2001. Distinct and overlapping functions of insulin and IGF-I receptors. *Endocr Rev* **22**(6): 818-835.

Nef, S., Shipman, T., and Parada, L.F. 2000. A molecular basis for estrogen-induced cryptorchidism. *Dev Biol* **224**(2): 354-361.

Nef, S., Verma-Kurvari, S., Merenmies, J., Vassalli, J.D., Efstratiadis, A., Accili, D., and Parada, L.F. 2003. Testis determination requires insulin receptor family function in mice. *Nature* **426**(6964): 291-295.

Neufeld, T.P., de la Cruz, A.F., Johnston, L.A., and Edgar, B.A. 1998. Coordination of growth and cell division in the Drosophila wing. *Cell* **93**(7): 1183-1193.

Nijhout, H. 1994. *Insect Hormones*. Princeton University Press, Princeton.

Nijhout, H.F. and Williams, C.M. 1974. Control of moulting and metamorphosis in the tobacco hornworm, Manduca sexta (L.): cessation of juvenile hormone secretion as a trigger for pupation. *J Exp Biol* **61**(2): 493-501.

Normann, T.C. 1975. Neurosecretory cells in insect brain and production of hypoglycaemic hormone. *Nature* **254**(5497): 259-261.

Noyes, B.E., Katz, F.N., and Schaffer, M.H. 1995. Identification and expression of the Drosophila adipokinetic hormone gene. *Mol Cell Endocrinol* **109**(2): 133-141.

Oldham, S. and Hafen, E. 2003. Insulin/IGF and target of rapamycin signaling: a TOR de force in growth control. *Trends Cell Biol* **13**(2): 79-85.

Oster, M.H., Levin, N., Fielder, P.J., Robinson, I.C., Baxter, R.C., and Cronin, M.J. 1996. Developmental differences in the IGF-I system response to severe and chronic calorie malnutrition. *Am J Physiol* **270**(4 Pt 1): E646-653.

Owen, O.E., Morgan, A.P., Kemp, H.G., Sullivan, J.M., Herrera, M.G., and Cahill, G.F., Jr. 1967. Brain metabolism during fasting. *J Clin Invest* **46**(10): 1589-1595.

Pan, D., Dong, J., Zhang, Y., and Gao, X. 2004. Tuberous sclerosis complex: from Drosophila to human disease. *Trends Cell Biol* **14**(2): 78-85.

Pende, M., Um, S.H., Mieulet, V., Sticker, M., Goss, V.L., Mestan, J., Mueller, M., Fumagalli, S., Kozma, S.C., and Thomas, G. 2004. S6K1(-/-)/S6K2(-/-) mice exhibit perinatal lethality and rapamycin-sensitive 5'-terminal oligopyrimidine mRNA translation and reveal a mitogen-activated protein kinase-dependent S6 kinase pathway. *Mol Cell Biol* **24**(8): 3112-3124.

Peng, T., Golub, T.R., and Sabatini, D.M. 2002. The immunosuppressant rapamycin mimics a starvation-like signal distinct from amino acid and glucose deprivation. *Mol Cell Biol* **22**(15): 5575-5584.

Petryk, A., Warren, J.T., Marques, G., Jarcho, M.P., Gilbert, L.I., Kahler, J., Parvy, J.P., Li, Y., Dauphin-Villemant, C., and O'Connor, M.B. 2003. Shade is the Drosophila P450 enzyme that mediates the hydroxylation of ecdysone to the steroid insect molting hormone 20-hydroxyecdysone. *Proc Natl Acad Sci U S A* **100**(24): 13773-13778.

Plisetskaya, E. 1989. Pancreatic peptides. in *The Comparative Physiology of Regulatory Peptides* (ed. S. Holmgren), pp. 174-202. Chapman and Hall, London.

Polymenis, M. and Schmidt, E.V. 1997. Coupling of cell division to cell growth by translational control of the G1 cyclin CLN3 in yeast. *Genes Dev* **11**(19): 2522-2531.

Poretsky, L., Cataldo, N.A., Rosenwaks, Z., and Giudice, L.C. 1999. The insulin-related ovarian regulatory system in health and disease. *Endocr Rev* **20**(4): 535-582.

Potter, C.J., Huang, H., and Xu, T. 2001. Drosophila Tsc1 functions with Tsc2 to antagonize insulin signaling in regulating cell growth, cell proliferation, and organ size. *Cell* **105**(3): 357-368.

Potter, C.J., Pedraza, L.G., and Xu, T. 2002. Akt regulates growth by directly phosphorylating Tsc2. *Nat Cell Biol* **4**(9): 658-665.

Radimerski, T., Montagne, J., Rintelen, F., Stocker, H., van der Kaay, J., Downes, C.P., Hafen, E., and Thomas, G. 2002. dS6K-regulated cell growth is dPKB/dPI(3)K-independent, but requires dPDK1. *Nat Cell Biol* **4**(3): 251-255.

Raught, B., Gingras, A.C., and Sonenberg, N. 2001. The target of rapamycin (TOR) proteins. *Proc Natl Acad Sci U S A* **98**(13): 7037-7044.

Rees, H.H. 1989. Zooecdysteroids: Structure and occurence. in *Ecdysone: From Chemistry to Mode of Action* (ed. J. Koolman), pp. 28-38. Thieme-Verlag, Stuttgart.

Reiling, J.H., Doepfner, K.T., Hafen, E., and Stocker, H. 2005. Diet-dependent effects of the Drosophila Mnk1/Mnk2 homolog Lk6 on growth via eIF4E. *Curr Biol* **15**(1): 24-30.

Reiling, J.H. and Hafen, E. 2004. The hypoxia-induced paralogs Scylla and Charybdis inhibit growth by down-regulating S6K activity upstream of TSC in Drosophila. *Genes Dev* **18**(23): 2879-2892.

Rintelen, F., Stocker, H., Thomas, G., and Hafen, E. 2001. PDK1 regulates growth through Akt and S6K in Drosophila. *Proc Natl Acad Sci U S A* **98**(26): 15020-15025.

Rodriguez, A., Zhou, Z., Tang, M.L., Meller, S., Chen, J., Bellen, H., and Kimbrell, D.A. 1996. Identification of immune system and response genes, and novel mutations causing melanotic tumor formation in Drosophila melanogaster. *Genetics* **143**(2): 929-940.

Rosenfeld, R.G. and Oh, Y. 1998. The blind men and the elephant--a parable for the study of insulin-like growth factor binding proteins. *Endocrinology* **139**(1): 5-7.

Rountree, D.B. and Bollenbacher, W.E. 1986. The release of the prothoracicotropic hormone in the tobacco hornworm, Manduca sexta, is controlled intrinsically by juvenile hormone. *J Exp Biol* **120**: 41-58.

Rulifson, E.J., Kim, S.K., and Nusse, R. 2002. Ablation of insulin-producing neurons in flies: growth and diabetic phenotypes. *Science* **296**(5570): 1118-1120.

Rusten, T.E., Lindmo, K., Juhasz, G., Sass, M., Seglen, P.O., Brech, A., and Stenmark, H. 2004. Programmed autophagy in the Drosophila fat body is induced by ecdysone through regulation of the PI3K pathway. *Dev Cell* **7**(2): 179-192.

Ruvinsky, I., Sharon, N., Lerer, T., Cohen, H., Stolovich-Rain, M., Nir, T., Dor, Y., Zisman, P., and Meyuhas, O. 2005. Ribosomal protein S6 phosphorylation is a determinant of cell size and glucose homeostasis. *Genes Dev* **19**(18): 2199-2211.

Röller, H., Dahm, K.H., Sweeley, C.C., and Trost, B.M. 1967. Die Struktur des Juvenilhormon. *Angew Chem* **79**: 190-191.

Saltiel, A.R. and Kahn, C.R. 2001. Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* **414**(6865): 799-806.

Sansal, I. and Sellers, W.R. 2004. The biology and clinical relevance of the PTEN tumor suppressor pathway. *J Clin Oncol* **22**(14): 2954-2963.

Sarbassov, D.D., Ali, S.M., Kim, D.H., Guertin, D.A., Latek, R.R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D.M. 2004. Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Curr Biol* **14**(14): 1296-1302.

Sarbassov, D.D., Guertin, D.A., Ali, S.M., and Sabatini, D.M. 2005. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* **307**(5712): 1098-1101.

Saucedo, L.J., Gao, X., Chiarelli, D.A., Li, L., Pan, D., and Edgar, B.A. 2003. Rheb promotes cell growth as a component of the insulin/TOR signalling network. *Nat Cell Biol* **5**(6): 566-571.

Scarborough, R.M., Jamieson, G.C., Kalish, F., Kramer, S.J., McEnroe, G.A., Miller, C.A., and Schooley, D.A. 1984. Isolation and primary structure of two peptides with cardioacceleratory and hyperglycemic activity from the corpora cardiaca of Periplaneta americana. *Proc Natl Acad Sci U S A* **81**(17): 5575-5579.

Schaffer, M.H., Noyes, B.E., Slaughter, C.A., Thorne, G.C., and Gaskell, S.J. 1990. The fruitfly Drosophila melanogaster contains a novel charged adipokinetic-hormone-family peptide. *Biochem J* **269**(2): 315-320.

Scott, R.C., Schuldiner, O., and Neufeld, T.P. 2004. Role and regulation of starvation-induced autophagy in the Drosophila fat body. *Dev Cell* **7**(2): 167-178.

Seecof, R.L. and Dewhurst, S. 1974. Insulin is a Drosophila hormone and acts to enhance the differentiation of embryonic Drosophila cells. *Cell Differ* **3**(1): 63-70.

Siegmund, T. and Korge, G. 2001. Innervation of the ring gland of Drosophila melanogaster. *J Comp Neurol* **431**(4): 481-491.

Simpson, L. and Parsons, R. 2001. PTEN: life as a tumor suppressor. *Exp Cell Res* **264**(1): 29-41.

Sloth Andersen, A., Hertz Hansen, P., Schaffer, L., and Kristensen, C. 2000. A new secreted insect protein belonging to the immunoglobulin superfamily binds insulin and related peptides and inhibits their activities. *J Biol Chem* **275**(22): 16948-16953.

Sokoloff, L. 1989. Circulation and Energy Metabolismof the Brain. in *Basic Neurochemistry* (ed. G. Siegel, B. Agranoff, R.W. Albers, and P. Molinoff), pp. 565-590. Raven Press, New York.

Steck, P.A., Pershouse, M.A., Jasser, S.A., Yung, W.K., Lin, H., Ligon, A.H., Langford, L.A., Baumgard, M.L., Hattier, T., Davis, T., Frye, C., Hu, R., Swedlund, B., Teng, D.H., and Tavtigian, S.V. 1997. Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat Genet* **15**(4): 356-362.

Steele, J.E. 1961. Occurence of a hyperglycaemic factor in the corpus cardiacum of an insect. *Nature* **192**: 680-681.

-. 1982. Glycogen phosphorylase in insects. *Insect Biochem* **12**: 131-147.

-. 1985. Control of metabolic processes. in *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (ed. Pergamon), pp. 99-145. G.A. Kerkut and L.I. Gilbert, New York.

Stewart, C.E. and Rotwein, P. 1996. Growth, differentiation, and survival: multiple physiological functions for insulin-like growth factors. *Physiol Rev* **76**(4): 1005-1026.

Stocker, H., Andjelkovic, M., Oldham, S., Laffargue, M., Wymann, M.P., Hemmings, B.A., and Hafen, E. 2002. Living with lethal PIP3 levels: viability of flies lacking PTEN restored by a PH domain mutation in Akt/PKB. *Science* **295**(5562): 2088-2091.

Stocker, H. and Hafen, E. 2000. Genetic control of cell size. *Curr Opin Genet Dev* **10**(5): 529-535.

Stocker, H., Radimerski, T., Schindelholz, B., Wittwer, F., Belawat, P., Daram, P., Breuer, S., Thomas, G., and Hafen, E. 2003. Rheb is an essential regulator of S6K in controlling cell growth in Drosophila. *Nat Cell Biol* **5**(6): 559-565.

Sulis, M.L. and Parsons, R. 2003. PTEN: from pathology to biology. *Trends Cell Biol* **13**(9): 478-483.

Tapon, N., Ito, N., Dickson, B.J., Treisman, J.E., and Hariharan, I.K. 2001. The Drosophila tuberous sclerosis complex gene homologs restrict cell growth and cell proliferation. *Cell* **105**(3): 345-355.

Tatar, M., Kopelman, A., Epstein, D., Tu, M.P., Yin, C.M., and Garofalo, R.S. 2001. A mutant Drosophila insulin receptor homolog that extends life-span and impairs neuroendocrine function. *Science* **292**(5514): 107-110.

Tremblay, F., Jacques, H., and Marette, A. 2005. Modulation of insulin action by dietary proteins and amino acids: role of the mammalian target of rapamycin nutrient sensing pathway. *Curr Opin Clin Nutr Metab Care* **8**(4): 457-462.

Twigg, S.M. and Baxter, R.C. 1998. Insulin-like growth factor (IGF)-binding protein 5 forms an alternative ternary complex with IGFs and the acid-labile subunit. *J Biol Chem* **273**(11): 6074-6079.

Uchida, T., Myers, M.G., Jr., and White, M.F. 2000. IRS-4 mediates protein kinase B signaling during insulin stimulation without promoting antiapoptosis. *Mol Cell Biol* **20**(1): 126-138.

Ullrich, A. and Schlessinger, J. 1990. Signal transduction by receptors with tyrosine kinase activity. *Cell* **61**(2): 203-212.

Um, S.H., Frigerio, F., Watanabe, M., Picard, F., Joaquin, M., Sticker, M., Fumagalli, S., Allegrini, P.R., Kozma, S.C., Auwerx, J., and Thomas, G. 2004. Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. *Nature* **431**(7005): 200-205.

Valentinis, B. and Baserga, R. 2001. IGF-I receptor signalling in transformation and differentiation. *Mol Pathol* **54**(3): 133-137.

Vanhaesebroeck, B., Leevers, S.J., Ahmadi, K., Timms, J., Katso, R., Driscoll, P.C., Woscholski, R., Parker, P.J., and Waterfield, M.D. 2001. Synthesis and function of 3-phosphorylated inositol lipids. *Annu Rev Biochem* **70**: 535-602.

Vogelstein, B. and Kinzler, K.W. 1993. The multistep nature of cancer. *Trends Genet* **9**(4): 138-141.

Vogt, P.K. 2001. PI 3-kinase, mTOR, protein synthesis and cancer. *Trends Mol Med* **7**(11): 482-484.

Wandji, S.A., Gadsby, J.E., Barber, J.A., and Hammond, J.M. 2000. Messenger ribonucleic acids for MAC25 and connective tissue growth factor (CTGF) are inversely regulated during folliculogenesis and early luteogenesis. *Endocrinology* **141**(7): 2648-2657.

Wang, X., Li, W., Williams, M., Terada, N., Alessi, D.R., and Proud, C.G. 2001. Regulation of elongation factor 2 kinase by p90(RSK1) and p70 S6 kinase. *Embo J* **20**(16): 4370-4379.

Weigmann, K., Cohen, S.M., and Lehner, C.F. 1997. Cell cycle progression, growth and patterning in imaginal discs despite inhibition of cell division after inactivation of Drosophila Cdc2 kinase. *Development* **124**(18): 3555-3563.

Weinkove, D., Leevers, S.J., MacDougall, L.K., and Waterfield, M.D. 1997. p60 is an adaptor for the Drosophila phosphoinositide 3-kinase, Dp110. *J Biol Chem* **272**(23): 14606-14610.

Weinkove, D., Neufeld, T.P., Twardzik, T., Waterfield, M.D., and Leevers, S.J. 1999. Regulation of imaginal disc cell size, cell number and organ size by Drosophila class I(A) phosphoinositide 3-kinase and its adaptor. *Curr Biol* **9**(18): 1019-1029.

White, M.F. 2002. IRS proteins and the common path to diabetes. *Am J Physiol Endocrinol Metab* **283**(3): E413-422.

Whiteman, E.L., Cho, H., and Birnbaum, M.J. 2002. Role of Akt/protein kinase B in metabolism. *Trends Endocrinol Metab* **13**(10): 444-451.

Wolfrum, C., Asilmaz, E., Luca, E., Friedman, J.M., and Stoffel, M. 2004. Foxa2 regulates lipid metabolism and ketogenesis in the liver during fasting and in diabetes. *Nature* **432**(7020): 1027-1032.

Wullschleger, S., Loewith, R., and Hall, M.N. 2006. TOR signaling in growth and metabolism. *Cell* **124**(3): 471-484.

Yamanaka, Y., Wilson, E.M., Rosenfeld, R.G., and Oh, Y. 1997. Inhibition of insulin receptor activation by insulin-like growth factor binding proteins. *Journal of Biological Chemistry* **272**(49): 30729-30734.

Zapf, J. 1995. Physiological role of the insulin-like growth factor binding proteins. *Eur J Endocrinol* **132**(6): 645-654.

Zhang, Y., Gao, X., Saucedo, L.J., Ru, B., Edgar, B.A., and Pan, D. 2003. Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. *Nat Cell Biol* **5**(6): 578-581.

Ziegler, R. 1979. Hyperglycaemic factor from the corpora cardiaca of Manduca sexta (L.) (Lepidoptera: Sphingidae). *Gen Comp Endocrinol* **39**(3): 350-357.

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Curriculum vitae

Name	Honegger
Vorname	Basil
Geboren	20. April 1977 in Zürich, Schweiz
Heimatort	Wald (ZH)
Nationalität	Schweizer

Ausbildung

Kantonsschule Hohe Promenade (ZH), Abschlussjahr 1997, Typus D

Biologiestudium von 1997-2002 an der Universität Zürich
(Diplomabschluss 2002)

Diplomfach: Zoologie (Entwicklungsbiologie bei Prof. Dr. Ernst Hafen),
Nebenfach: Molekularbiologie

Titel der Diplomarbeit: Characterization of the Imaginal Morphogenesis Protein-Late 2 (Imp-L2) – a negative regulator of the Insulin Signaling Pathway in *Drosophila melanogaster*
(Leitung Prof. Dr. Ernst Hafen)

an der Universität Zürich seit 1997